

**EFFECTS OF EDIBLE BIRD'S NEST ON ENDOMETRIAL RECEPTIVITY
AND FERTILITY IN RATS SUBJECTED TO INDOMETHACIN AND LEAD
ACETATE TOXICITY**

By

SOLANGI MARIA AMIR

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

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The Endocrine disrupting chemicals (EDCs) can be derived from various sources which include pharmaceutical products and toxic heavy metals as environmental pollutants such as lead (Pb). Indomethacin (IMC) used as nonsteroidal anti-inflammatory drug (NSAID) is a pharmaceutical product reported to be embryotoxic leading to disruption of implantation process when given at doses higher than the therapeutic limit. Edible bird's nest (EBN), produced by Swiftlets (*Aerodramus fuciphagus*), considered in this study due to its composition and bioactive properties as antioxidative, and promoting cell-growth. The main objectives of the present study were to determine the optimal dose of IMC to cause embryo implantation dysfunction in Sprague Dawley (SD) rats without compromising their overall health, and to assess EBN's protective role against IMC's and PbA's toxicity effect on reproductive function and pregnancy.

Effect of EBN in its raw form supplement on EID induced by IMC toxicity was assessed. A total of 30 (6x5 groups) of SD rats (untreated control, a positive control with IMC (4.33 mg/kg bwt), and 3 treatment groups with same IMC dose and EBN (60, 90, and 120 mg/kg bwt) were recruited. The next phase of the study was aimed at evaluating the prophylactic role of EBN supplement against PbA toxicity on pregnant rats' ovarian and uterine functions. Two forms of EBN, raw and hydrolysed (REBN & HEBN) were used. The experimental setting was similar with that of the previous study, except for the toxic agent used here is PbA (10 mg/kg bwt) and additional replicates for HEBN used at doses of 20, 40, and 60 mg/kg bwt, given orally for a period of 8 weeks.

In summary, the findings revealed leads to the conclusion that IMC (at a dose of 4.33 mg/kg bwt) can be used to deter embryo implantation in SD rats with minimal damage to vital organs for reproductive toxicity studies. EBN counteracts the harmful effects and protects the reproductive system in a multimodal mechanism by modulating redox balance, enhanced production steroid hormones and expressions of growth factors as well as promoting cell growth and proliferation. The reduction in MT expression and Pb deposition in ovarian and uterine tissues along with higher EBN dose supplementation might reflect the potential role of EBN as chelating agent in the gut as well as enhancing detoxification and excretion of Pb by the vital organs such as liver and kidney, requiring further study to verify. Comparing the two forms of EBN (REBN & HEBN), doses of 90 & 120 mg/kg bwt/day of REBN and 60 mg/kg bwt/day performed best while 90 and 120 mg/kg bwt/day of REBN appears to be most superior considering all the variables studied. Lastly, the observed findings on EBN as supplement to enhance reproduction coincides with one of the reasons of consumption of EBN.

Keyword: Edible Bird Nest, Growth Factors, Indomethacin Uterus, Lead Toxicity, Ovaries, Oxidative Stress and Steroid Hormones.

SDG: GOAL 3: Good Health and Well-Being



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**KESAN SARANG BURUNG YANG BOLEH DIMAKAN (EBN) KE ATAS
PENERIMAAN DAN KESUBURAN ENDOMETRIUM TIKUS YANG
TERDEDAH KEPADA KETOKSIKAN INDOMETASIN DAN PLUMBUM
ASETAT**

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Bahan kimia pengganggu endokrin (EDC) boleh diperoleh daripada pelbagai sumber termasuk produk farmaseutikal dan logam berat toksik sebagai bahan pencemar alam sekitar seperti plumbum (Pb). Indomethacin (IMC) yang digunakan sebagai ubat anti-radang bukan steroid (NSAID) adalah produk farmaseutikal yang dilaporkan bersifat embriotoksik yang membawa kepada gangguan proses implantasi apabila diberikan pada dos yang lebih tinggi daripada had terapeutik. Pencarian produk semula jadi untuk digunakan sebagai makanan tambahan bagi mengurangkan kesan toksik jenis EDC adalah amat penting. Sarang burung yang boleh dimakan (EBN) yang dihasilkan oleh burung Walit (*Aerodramus fuciphagus*), dianggap sebagai bahan yang berpotensi dalam kajian ini kerana komposisinya dan sifat-sifat bioaktifnya seperti antioksidan, dan penggalak pertumbuhan sel dan percambahan. Selain itu, kajian tentang peranan perlindungan EBN terhadap IMC dan plumbum asetat (PbA) adalah terhad. Oleh itu, objektif utama kajian ini adalah untuk menentukan dos optimum IMC yang boleh menyebabkan disfungsi implantasi embrio pada tikus Sprague Dawley (SD) tanpa

menjejaskan kesihatan keseluruhan tikus, dan untuk menilai peranan perlindungan EBN terhadap kesan ketoksikan IMC dan PbA terhadap fungsi pembiakan. dan kehamilan.

Seterusnya, kesan perlindungan EBN dalam bentuk mentah pada EID yang disebabkan oleh ketoksikan IMC telah dinilai. Sebanyak 30 (6x5 kumpulan) tikus SD (kawalan tidak dirawat, kawalan positif dengan IMC (4.33 mg/kg bwt), dan 3 kumpulan rawatan dengan dos IMC yang sama dan EBN (60, 90, dan 120 mg/kg bwt)) telah diambil. Fasa kajian yang seterusnya bertujuan untuk menilai peranan profilaksis suplemen EBN terhadap ketoksikan PbA pada fungsi ovari dan rahim tikus hamil. Dua bentuk EBN, mentah dan terhidrolisis (REBN & HEBN) telah digunakan. Tetapan eksperimen adalah serupa dengan kajian terdahulu menggunakan IMC, kecuali agen toksik yang digunakan di sini ialah PbA (10 mg/kg bwt) dan replika tambahan untuk HEBN yang digunakan pada dos 20, 40, dan 60 mg/kg bwt, diberikan secara lisan untuk tempoh 8 minggu. Secara ringkasnya, penemuan yang didedahkan membawa kepada kesimpulan bahawa IMC (pada dos 4.33 mg/kg bwt) boleh digunakan untuk menghalang implantasi embrio dalam tikus SD dengan kerosakan minimum pada organ penting untuk kajian ketoksikan pembiakan. EBN mengatasi kesan berbahaya dan melindungi sistem pembiakan dalam mekanisme multimodal dengan memodulasi keseimbangan redoks, meningkatkan pengeluaran hormon steroid dan ekspresi faktor pertumbuhan serta menggalakkan pertumbuhan dan percambahan sel. Pengurangan dalam ekspresi MT dan pemendapan Pb dalam tisu ovari dan rahim bersama dengan suplemen dos EBN yang lebih tinggi mungkin mencerminkan potensi peranan EBN sebagai agen pengkelat dalam usus serta meningkatkan detoksifikasi dan perkumuhan Pb oleh organ penting seperti hati dan buah pinggang, dan ini memerlukan kajian lanjut

untuk pengesahan. Perbandingan dua bentuk EBN (REBN & HEBN), dos 90 dan 120 mg/kg bwt/hari REBN dan 60 mg/kg bwt/hari menunjukkan prestasi terbaik manakala 90 dan 120 mg/kg bwt/hari REBN nampaknya paling terunggul mengambil kira semua pembolehubah yang dikaji. Akhir sekali, penemuan yang diperhatikan mengenai EBN sebagai suplemen tambahan untuk meningkatkan pembiakan bertepatan dengan salah satu sebab penggunaan EBN oleh manusia.

Kata kunci: Faktor Pertumbuhan, Ketoksikan Plumbum, Ovari, Sarang Burung Boleh Makan, Tekanan Oksidatif dan Hormon Steroid, Uterus Indomethacin.

SDG: MATLAMAT 3: Kesihatan dan Kesejahteraan Yang Baik

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LIST OF ABBREVIATIONS

AF	Atretic follicle
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
AO	Antioxidants
BBL	Blood lead level
bwt	Body weight
Caco-2 cells	Human colonic adenocarcinoma cell line
CL	Corpus luteum
CO ₂	Carbon dioxide
Cpm	Counts per minute
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
E ₂	Oestrogen
E ₂ R	Receptor of oestrogen
EBN	Edible bird's nest
EDCs	Endocrine disrupting chemicals
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EID	Embryonic implantation dysfunction
EIR	Embryonic implantation rate
F	Follicular unit
FGFS	Fibroblast growth factors
FSH	Follicle-stimulating hormone
GE	Glandular epithelium
GH	Growth hormone
GSH	Glutathione

H and E	Haematoxylin and Eosin
H ₂ O ₂	Hydrogen peroxide
ICM	Inner cell mass
ICP-MS	Inductive coupled plasma mass spectrometry
IGF	Insulin-like growth factor
IL-6	Interleukin 6
IMC	Indomethacin
IN	Interstitial cell
LE	Luminal epithelium
LH	Luteinizing hormone
MT	Metallothioneins
m	Uterine muscle atrophy
NC	Control stain without antibody
NF	Antral follicle
NOS	Nitrogen oxide species
O ₂ -	Superoxide
OBWR	Ovarian body weight ratio
OS	Oxidative stress
OSB	Oxidative stress biomarkers
OSE	Ovarian surface epithelium
P ₄	Progesterone
P ₄ R	Receptor of Progesterone
Pb	Lead
PCNA	Proliferating cell nuclear antigen
ROS	As reactive oxygen species
S	Stromal cells
SA	Sialic acid
SE	Standard error

SEM	Scanning electron microscope
SOD	Superoxide dismutase
TAC	Total antioxidant capacity
TBARS	Thiobarbituric acid reactive substance
UBWR	Uterine body weight ratio
UE	Uterine endothelium
u	Number of uterine glands in endothelium
VEGF	Vascular endothelial growth factor





CHAPTER 1

INTRODUCTION

1.1 Overview

As early as the Tang Dynasty, edible bird's nest (EBN) was considered a premium health food and a symbol of ancient aristocrats' status due to its scarcity, flavour, and nutritional value (Dai et al., 2021). Chinese consumers worldwide like EBN health foods. The White nest swiftlet (*Aerodramus fuciphagus*) and Black nest swiftlet (*A. maximus*) are heavily exploited and produce most EBNs traded worldwide. Swiftlets, small insectivorous Apodidae birds of the Collocaliini tribe, fly over the western Indian Ocean Islands, Southern Asia, the Philippines, North-Western Australia, Western Australia, and the Southwest Pacific. Currently, there are 24 Swiftlet species have been discovered all over the world (Lee et al., 2021). According to (Hobbs & Conservation, 2004), Indonesia produced the most swiftlets, followed by Sarawak and Sabah in Borneo (Jamaluddin et al., 2019). Malaysian swiftlets are mostly *A. fuciphagus*. It uses two pairs of salivary glands behind its tongue to generate sticky saliva that resembles starch to build its nest and protect its young (Hobbs & Conservation, 2004).

In Chinese literature, EBN is called "Yan Wo"—"swift's nest." Bird's nest and sugar were double boiled to make a powerfully nutritious soup and treat medicines. Bird's nest soup was thought to improve skin and appearance. Jamalluddin et al. (2019) reported EBN was provided for therapeutic and medicinal purposes (Jamalluddin et

al., 2019). EBN provides high-quality functional proteins and structurally diverse dietary components (Babji et al., 2018). EBN combat cancer, ageing, phlegm, cough, TB, voice improvement, general debility and asthenia, and illness and operation recovery. Pharmacological and nutritional secrets of EBN have been extensively studied (Lee et al., 2021).

According to the Chinese Compendium of Materia Media, EBN reduces asthmatic symptoms, boosts the immune system, reduces stomach aches, and increases endurance. Numerous studies have shown that the water-soluble extract of EBN contains EGF) after soaking, prevents influenza virus infection, reduces oxidative stress in vitro, and induces mitosis in human peripheral blood monocytes and adipose-derived stem cells. Recent research show that hot water extract increases rabbit corneal keratocyte proliferation (Kong et al., 2016). VEGF, EGF, and Interleukin (IL-6) help cells communicate. These substances mediate cell survival, proliferation, differentiation, and effector activity. IL-6 cytokines help embryonic and adult stem cells survive. When exposed to IL-6, placental MSCs proliferate. In response to hypoxia, human adipose stromal cells release growth factors including VEGF and HGF, which enhance cell proliferation and decrease cell death in conditioned medium (Rehman et al., 2004; Roh et al., 2012).

Antioxidants are popular because they may treat oxidative stress-related diseases, which are predicted to develop worldwide. Oxidative stress is linked to most chronic diseases, and antioxidants can reduce risk and control sickness. Despite high antioxidant content, nutrkinetic variables may impact bioactive absorption from dietary sources and bioactivity (Yida et al., 2014). Enzymatic and non-enzymatic

antioxidant defences protect the body from oxidants and disease-causing free radicals. Chemical synthesis and plant and animal tissues produce antioxidants. Glycoproteins, the main component of EBNs, are linked to health and beauty. EBN has several proteins, amino acids, and mineral salts. EBN is protein-rich and contains all essential amino acids, including valine, phenylalanine, threonine, and proline (Ali et al., 2019).

Alcalase produces antioxidant EBN glycopeptides. According to amino acid composition, house EBN glycopeptides with strong antioxidant properties had more tyrosine, histidine, tryptophan, and phenylalanine than glycoprotein/unhydrolyzed EBN. However, cave EBN glycopeptides' antioxidant activity was strongly correlated with their hydrophobic and aromatic amino acid residues (Daud et al., 2021).

Recent research reveals that EBN is anti-inflammatory and antioxidant, however the mechanisms are unknown. In vitro findings may not represent in vivo situations. Synergistic effects also explain the bioactivity of multi-ingredient diets. EBN contains proteins, carbohydrates, fatty acids, and minerals. One study related EBN's bioactivity to its 11% protein component, sialic acid (Ali et al., 2019; Lee et al., 2021). EBN increases cellular communication pathways like EGF, VEGF, and IL-6. Intercellular mediators regulate cell proliferation, survival, effector duties, and differentiation. IL-6 controls embryonic, adult, and multipotent MSC development. Although one of the widely held benefits of EBN and one of the drivers driving EBN intake, the stated benefit has not been scientifically proven. EBN enhances oestrogen synthesis and acts as an antioxidant, according to new study in unrelated domains, EBN contained testosterone, oestradiol (E2), progesterone (P4), LH, FSH, and prolactin (Ma et al., 2012; Albishtue et al., 2018) . These hormones regulate mammalian reproduction (Ma et al., 2012).

EBN also estrogenically stimulates uterine glands. Oestrogen induces the P4 gene to increase progesterone activity and uterine epithelial proliferation. The female reproductive system needs oestrogen to prevent menstrual dysfunctions such as amenorrhea and irregular menstruation. Albishtue (2018) found that uterine weight and length affect EBN oestrogen and sialic acid levels (Albishtue et al., 2018). EBN protects uterine glandular secretions like growth factors, hormones, and transport proteins needed for conception (Albishtue et al., 2019).

Industrial pollutants damage the environment and harm reproductive mammals (Zhang et al., 2016; Ideta-Otsuka et al., 2017; Alves et al., 2018; Nerin et al., 2018). Heavy metals and chemicals harm mammalian reproduction. Lead (Pb) is a persistent heavy metal that affects the environment, mammalian reproductive, and biochemical, behavioural, and physiological dysfunctions (Singh et al., 2010; Sofoulaki et al., 2019; Karri et al., 2020). Data from the Institute for Health Metrics and Evaluation, which was recently mentioned by the World Health Organisation (WHO) (2024), indicates that 21 million disability-adjusted life years have been lost as a result of lead exposure's long-term health effects. Furthermore, they determined that exposure to lead was responsible for 30% of idiopathic developmental problems and 3% of the global burden of chronic renal illnesses. Pb significantly affects the hypothalamic-pituitary axis, decreasing GH production and somatotrope receptivity (Ahmed et al., 2013; Protsenko et al., 2018; Shojaeepour et al., 2018). It also raises blood prolactin, affects thyroid hormone function, and disturbs the calciotropic hormone balance, which are linked to bone diseases (Benoff et al., 2000). Lead acetate (PbA) is equally toxic since it accumulates in animal tissues and has a lengthy half-life. PbA poisoning increases ROS, NOS, and OS (Haleagrahara et al., 2010; Liu et al., 2014; Rzymiski et al., 2014).

Lead exposure damages the liver, pituitary gland, and reproductive system, including the ovary, uterus, and ovarian follicles (Bretveld et al., 2006). Reactive oxygen species and antioxidant deficiency cause PbA toxicity. PbA can pass bio membranes and precipitate in the ovary and placenta, causing poisoning. Long-term lead exposure lowers progesterone, luteinizing hormone, and follicle-stimulating hormone levels in primates, decreasing ovarian and luteal activity. Lead decreases primordial and increases atretic follicles in mouse ovaries. It affects mice uterine glands, columnar cell height, endometrium, myometrium, and perimetrium (Nakade et al., 2015). Qureshi and Sharma identified degenerative and inflammatory alterations in the uterus, including reduced columnar cell height and undetected blood arteries, lymphatics, and connective tissues (Qureshi et al., 2012). Lead exposure in utero or during pregnancy has been linked to uterine gland necrosis and the death of uterine lining cells. Lead exposure during pregnancy can cause uterine gland necrosis and uterine lining cell loss. Endometrial gland secretions alter oestrous cycles, conceptus survival, and peri-implantation growth (Gray et al., 2002). Lead may thereby affect the uterine glands' secretions, which comprise enzymes, growth factors, cytokines, lymphokines, hormones, and transport proteins essential for the development of the foetus and the conceptus, so interfering with in-utero physiological processes (Spencer et al., 2004; Nakade et al., 2015).

1.2 Problem statement

In rats, implantation can only take place during a brief window of time called the limited period, which typically lasts between 4 and 8 days into the pregnancy (Enciso

2021). There are three stages of uterine implantation: the pre-receptive phase, which occurs one to three days after fertilization; the receptive stage, which occurs four days after conception; and the non-receptive stage (after fertilization) (Soni et al., 2020). The implantation of an embryo is something that can only take place during the receptive phase; the procedure cannot be finished in either of the other two phases. During 12 to 14 days, it is possible to make an accurate diagnosis of an abortion (Shan et al., 2019). The embryo implantation dysfunction (EID) was induced by a number of factors, both extrinsic and intrinsic. The most recent research has demonstrated that lead (Pb) can alter the prooxidant/antioxidant balance in tissue, which can lead to a variety of dysfunctions in both essential organs and reproductive organs (Albishtue et al., 2018) .

Lead (Pb), a widespread and potent hazardous toxin, causes a number of physiological as well as behavioural variations. Lead also impairs the operation of many organs and related systems, most notably the reproductive system. Pb can cause oxidative stress, barrier disruption, and change various Ca^{2+} -dependent processes in development and adult animals, including physiological processes involving nitric oxide generation (Ferizi et al., 2020). Natural antioxidants are very beneficial in this case. Lead poisoning is an old but persistent public health issue in underdeveloped countries (Showell et al., 2014; Albishtue et al., 2018) . It has been determined that malnutrition, ovulatory or hormonal abnormalities, and infectious agents are the primary causes of infertility and the subsequent losses in productivity that occur in milk-producing animals because of these conditions. Nevertheless, there is little information available on how environmental contaminants affect female reproductive health and how this affects reproduction. Harmful effects of Pb on male reproduction, including altered

spermatogenesis, increased sperm pathologies, and testicular degeneration, have been extensively researched. However, studies on the toxicology of female reproduction are scarce and differ from those on male reproduction due to differences in gametogenesis, access to germinal cells, and the fluidity of female reproductive function. Infant mortality, miscarriage, abortion, early birth, and severe lead poisoning have all been associated. Lead enters the placenta during pregnancy and has been associated with intrauterine death, preterm birth, and low birth weight (Nakade et al., 2015; Albishtue et al., 2018). Prolonged Pb exposure in laboratory animals may impair follicular development, ovulation, and menstruation in monkeys, delay pubertal rat vaginal opening, and reduce the rate at which mouse eggs are implanted and subsequent litters are born (Qureshi et al., 2012; Nakade et al., 2015; Albishtue et al., 2020).

Studies on EBN's influence outside of toxicity, however, have uncovered significant biological characteristics evidence of EBN's potential ability to reduce heavy metal toxicity. Despite EBN's impressive antioxidant credentials, it is unclear whether this means that Pb poisoning will have less of an impact on the liver and kidney (Albishtue et al., 2020; Quddus et al., 2021). According to recent research, EBN promoted uterine structure proliferation and differentiation, which (as seen by elevated steroid receptor expression) promoted fertility and embryo implantation in lead-acetate-exposed rats (Albishtue et al., 2019). The makeup of EBN is also said to include progesterone (P4), testosterone, oestradiol (E2), LH, FSH, and prolactin, all of which are reproductive hormones. Oestradiol, a key EBN extract contains oestrogen, a hormone involved in reproduction. This kind of oestrogen is the most prevalent and is crucial for the female reproductive system. EBN offers a number of therapeutic benefits and has been shown to affect anti-inflammatory and antioxidant genes (Yida et al., 2015; Jaffar et al.,

2021). Yet, only a small amount of study has identified EBN's fertility-enhancing qualities against the harmful effects of heavy metals on mammalian reproduction and fertility (Ma et al, 2012). Although EBN has been hailed as a powerful antioxidant, it is unclear whether it can help to mitigate consequences of PbA toxicity on reproductive system and the vital organs associated (Doumouchtsis et al., 2009; Yida et al., 2014).

Hydrolyzed EBN and raw EBN have not been studied in terms of their potential protective effect against PbA-facilitated reproductive damage in female rats. Tests were conducted to see which one was more effective at reducing lead acetate's toxic effects on reproduction and other organs in pregnant rats. Research on how EBN supplement affects fertility and reproduction is scarce, despite the possible biological effects that EBN may have (Albistue et al., 2019). Endometrial and breast cancer may occur as a result of numerous different types of hormone replacement therapy (Jiang et al., 2024), but EBN has been commended for its great potential to be utilised as a prophylactic hormonal replacement therapy without any documented negative effects.

1.3 Objectives

General objectives

To evaluate the role of EBN in mitigating reproductive toxicity cross by IMC and PbA using a rat model

Specific objectives

1. To establish embryo implantation dysfunction and evaluate histopathological changes in vital organs induced by indomethacin in rat model.

2. To assess prophylactic effect of EBN against indomethacin induced embryo implantation dysfunction in rats.
3. To evaluate the ameliorating effect of raw and hydrolysed EBN against lead acetate toxicity on pregnancy and implantation rate, uterine and ovarian tissue.
4. To determine the ameliorating effect of raw and hydrolysed EBN towards lead acetate toxicity based on antioxidant (SOD), expressions of growth factors, steroid hormones and metallothionein (MT) in uterine, ovarian and vital organs.

1.4 Research hypothesis

Objective 1:

H_0 = Indomethacin (IMC) toxicity has no significant effect against embryo implantation and no histomorphological changes were observed in uterine and ovarian, kidney and liver tissues.

H_a = IMC toxicity have significant effect on embryo implantation and histomorphological changes were observed in uterine and ovarian, kidney and liver tissues.

Objective 2:

H_0 = EBN supplement has no prophylactic effect against embryo implantation dysfunction (EID) caused by IMC toxicity.

H_a = EBN supplement has significant prophylactic effect against embryo implantation dysfunction (EID) caused by IMC toxicity.

Objective 3:

H_0 = raw and hydrolysed (R and HEBN) supplement has no significant ameliorating effect in pregnancy and implantation rate, uterine and ovarian tissue caused by PbA toxicity.

H_a = raw and hydrolysed (R and HEBN) supplement have significant ameliorating effect on pregnancy and implantation rate, uterine and ovarian tissue caused by PbA toxicity.

Objective 4:

H_0 = R and HEBN supplement has no substantial attenuating influence on the expressions of metallothionein on the uterine, ovarian, kidney and liver tissues of the PbA-intoxicated rats.

H_a = R and HEBN supplement have significant attenuating influence on the expressions of metallothionein on the uterine, ovarian, kidney and liver tissues of the PbA-intoxicated rats.



CHAPTER 2

LITERATURE REVIEW

2.1 The reproductive anatomy and physiology of female rats

The rat is a common experimental animal used to examine reproductive function. The female reproductive system is also known as the gonads (Figure 2.1). The genitourinary system is made up of the uterus, oviducts, fallopian tubes, and vagina. The ovarian bursa, which surrounds the ovary, is produced by this ostium in rats and is a fully developed capsule. The oviducts are little tubes that are tightly wrapped. The uterus has two unique uterine horns, which enable the rat to have several offspring (Huda & Md, 2013).

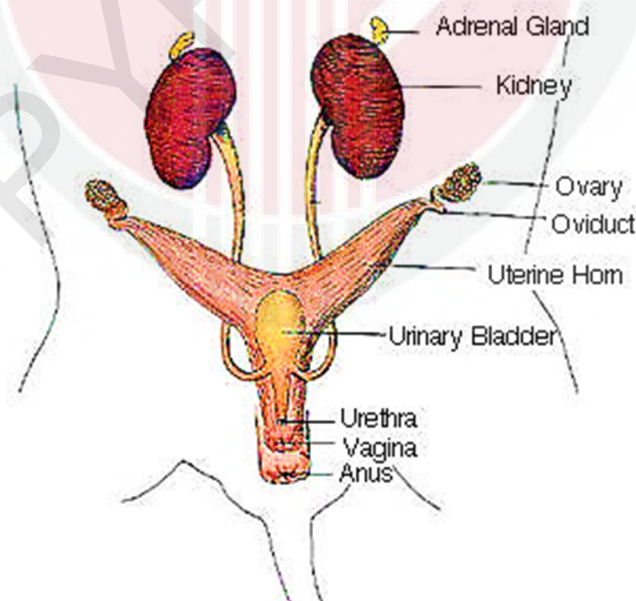


Figure 2.1: The Reproductive Organs of the Female Rat

[Retrieved from (https://www.biologycorner.com/worksheets/rat_urogenital.html)]

2.1.1 Oestrous cycle and mating

There are only four to five days in the rat oestrous cycle. It occurs all year round and is not affected by the seasons. The oestrous cycle depends on hormones and in rats it is short (4-5 days) and consists of four stages as proestrus, oestrus, metestrus and diestrus. The ovary develops pre- and peri-ovulatorily during the proestrus, which results in the synthesis and secretion of oestrogens when the exterior structure is swollen and wet. Oestrus is the brief period of time when the female accepts the male and ovulation takes place when the vulva's enlargement is visible from the outside. Progesterone is the primary hormonal effect during the early luteal phase, which is pale and dry with a slough of white cellular debris during metestrus. Diestrus features a highly wet vaginal opening. In the exfoliative cytology of the mouse vagina, polymorphonuclear leukocytes and squamous epithelial cells are the two main cell types (Bertolin & Murphy, 2014). These stages of the oestrous cycle are accompanied by physical changes of the reproductive organs, both internally and externally are shown in (Figure 2.2). Gonadotrophins are secreted by the anterior pituitary gland, and they work with hormone (FSH) and luteinizing hormone (LH) to regulate the oestrous cycle (FSH). Hormonal swings are responsible for abnormalities in the ovaries, follicles, and vaginal cytology. Follicle growth is accelerated by FSH, but ovulation and corpus luteum development are sped up by LH. During metestrus, corpus luteum secretes progesterone, which decreases during dioestrus. Oestradiol-17 (E2) steroid hormone is the ovary, it promotes the growth of the uterine endometrium, triggers the release of LH before ovulation, and blocks the release of pituitary hormone through negative feedback, its levels rise during follicular development. Estrogen contributes to regulating female reproductive health, maturation, and function. The cycle concludes when oestrogen reaches a high during proestrus, boosting gonadotropin release and triggering ovulation. (Ajayi et al., 2020; Huda & Md, 2013; Westwood,

2008). Between the start of proestrous and the end of oestrous, ovulation takes place. During the proestrous phase, prolactin, LH, and FSH levels all peak in the afternoon and stay elevated throughout the entire oestrous cycle. Oestradiol levels start to increase during the metestrous, reach their peak during the proestrous, and then return to normal during the oestrous. During the metestrous and the subsequent dioestrous, progesterone output rises. The second peak of progesterone is then reached near the conclusion of proestrous (Marcondes et al., 2002; Westwood, 2008); Ajayi et al., 2020). Oestrous cycles in rats and mice last 4-5 days, with 4 days being the most typical length in Sprague Dawley rats (Pestana & Graham, 2024).

Complex mating behaviour in rats may affect reproductive success. Mounts, intromissions, and ejaculations comprise rat mating (Albishtue et al., 2018; Frye & Chittur, 2020).

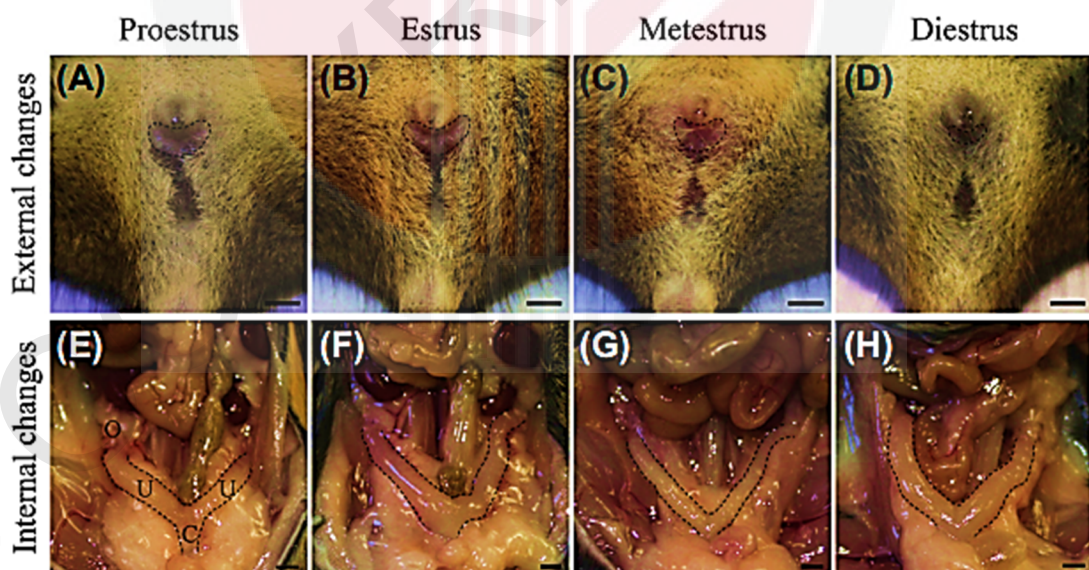


Figure 2.2: Visual guide to the mouse oestrous cycle depicting external and internal changes during (A/E) Proestrous, (B/F) Estrous, (C/G) Metetrous and (D/E) Diestrous
(Bertolin & Murphy, 2014)

2.1.2 Oestrous synchronization and pregnancy detection

Oestrous synchronisation, which can take place at any time of year and shortens the reproductive cycle, enables an increase in offspring each year. Consistent delivery timings are also made possible throughout the animal production unit. Oestrous synchronisation for artificial insemination or natural mating is made possible with the use of hormone agents, which can increase animal reproductive success. Another strategy for reducing reproduction is the use of prostaglandins, which dissolve the corpus lutein and cause the ovulation that starts the subsequent follicular phase (Wei et al., 2015). Not every female rat that mates with another in a lab environment gives birth to offspring. The likelihood of becoming pregnant varies and may even be very low. The main cause of the low pregnancy rate is because the female rats may not have been in an Oestrous state when they mated, which would have prevented successful fertilisation. Regrettably, it is challenging to predict and identify pregnancy success or failure in the early stages of gestation. A vaginal plug does not indicate that the female rat is pregnant or even that her bwt has grown; it only indicates that copulation has taken place. To increase the likelihood of a successful pregnancy, a variety of oestrous synchronisation strategies can be applied. The practise of controlling an animal's reproductive cycle by using hormones or their analogues to trigger ovulation at a certain period is known as hormonal synchronisation of ovulation (Smarr et al., 2016; Albishtue et al., 2018; Kurniawan Gondo, 2019).

Pregnancy in rats is indicated by belly growth (day 13), mammary development (day 14), nipple enlargement (day 14), and increased bwt and food consumption, and is confirmed by birth (Ypsilantis et al., 2009; Albishtue et al., 2018; Albishtue et al.,

2020). For the purpose of early rat pregnancy identification, a manual palpation technique has been developed. The animal is softly restrained by one hand, which is holding it as normal from behind. The first three fingers of the other hand are on the ventral midline, while the thumb is positioned on the posterior abdomen. The three fingers were gently pressed laterally and upward. Next, using the thumb, three fingers, and abdominal wall, the embryos can be felt. This palpation technique had no effect on the number of foetuses. With the help of this method, pregnancy could be identified with an accuracy rate of 80% on the fifth day of pregnancy and 100% between the sixth and ninth days (Albishtue et al., 2018). Serum luteinizing hormone (LH) levels are said to peak in the middle of pregnancy, decline during pregnancy, and then rise again at the end of gestation. Serum LH concentrations in rats were shown to be higher during the first 11 days of pregnancy than between days 13 and 19 (Sarvestani et al., 2014). Day 1 of gestation is defined as the day sperm are found in the vaginal smear. Foetuses can be palpated as early as day 10 of pregnancy, though accuracy improves after day 12. A pregnant woman's 13th day, there is significant abdominal growth, and by day 14, there is visible breast development and nipple enlargement (Huda & Md, 2013; Albishtue et al., 2018; Albishtue et al., 2020).

2.1.3 Fertilization and embryonic development

The intricate process of fertilisation involves several phases and the interaction of spermatozoa and eggs. Spermatozoa attaching to the ova's zona pellucida is the initial stage. Subsequently, a protein located in the spermatozoa's acrosome penetrates, causing the spermatozoa to fuse with the egg and initiate mitotic divisions, ultimately resulting in the formation of morula on the fourth day. A blastocyst is formed by

successive mitotic divisions. After differentiating, a blastocyst becomes a layer of trophoblast cells. The trophoblast cells gave rise to the placenta and inner cell mass (ICM), which gave rise to the embryo (Nordhoff & Wistuba, 2023).

After fertilisation, the zygote divides into two cells on day one, four cells on day two, eight cells on day three, and a sixteen-cell embryo on day four. On day four of pregnancy, the embryo develops into a morula, and on day five, it becomes a blastocyst. From the female reproductive system, pre-implantation rat embryos can be obtained and used for fundamental research (Cohen et al., 2000; Lee et al 2004; Huda et al 2013; Agca 2020) .

2.2 Embryonic implantation and dysfunction

Apposition, adhesion (attachment), and invasion are the three steps of implantation. The blastocyst is in close proximity to the luminal epithelium when the uterine lumen closes after the loss of the zona pellucida. The "implantation window" refers to the time frame within which embryos can implant in the uterus (Namiki et al., 2018). The uterine tissues that surround and oppose the embryo change cellularly in response to peri-implant signals. This process, known as decidualisation, is important for invasion and aids in the transfer of nutrients (Murata et al., 2022). The uterine stromal site surrounding the embryo divides its cells in response to embryonic signals, forming an organised vascular primary decidual zone. Meanwhile, the cell layer adjacent to the main decidual zone multiplies and differentiates, forming a secondary vascularised decidual zone.

Decidua is crucial for promoting the passage of nutrients, minimising maternal rejection during invasion, and facilitating this process. The uterine stromal site surrounding the embryo divides its cells in response to embryonic signals, forming an organised vascular primary decidual zone. Meanwhile, the cell layer adjacent to the main decidual zone multiplies and differentiates, forming a secondary vascularised decidual zone. Decidua is crucial for promoting the facilitating of nutrients, minimising maternal rejection during invasion (Moffett & Shreeve, 2023).

The implantation process involves several molecules, all of which must engage with the developing foetus and mother the confines of the implantation window. Pregnancy outcome, birth outcomes, such as sex ratio, live birth, birth weight, spontaneous abortion, low birth weight, early delivery, or stillbirth (Oyesola et al., 2019).

2.2.1 Uterine changes during implantation

In order to create room for the growing foetus, the uterus undergoes a series of carefully regulated morphological changes throughout pregnancy. In response to blastocyst implantation, fibroblast-like stromal cells in the endometrium of rats proliferate and differentiate into decidual cells, forming the decidua. The major decidual zone in the rat uterus is formed by antimesometrial decidual cells, which are generated at the antimesometrial pole of the uterine endometrium near the implanting blastocyst. The antimesometrial decidua, as it is now known, has attained its maximal development by day 10 and the decidual reaction has reached the basal zone near the circular muscle coat by day 8. After that, it goes backward to form the decidua capsularis. The mesometrial decidua forms on day 8 as differentiation spreads to the mesometrial side of the endometrium. The peak growth of this tissue occurs on day 12

of pregnancy (Pampfer et al., 1999; Welsh & Enders, 1991; Correia-da-Silva et al., 2004).

Endometrial stromal cells (SCs) are part of the uterine lining and are found between the luminal epithelium (LE) and the glandular epithelium (GE). The main hormones that control the alterations in uterine compartments are oestrogen and progesterone (P4), which are important in SC proliferation and epithelial cell repression through the expression of Indian hedgehog homolog (IHH) and heart- and neural crest derivatives-expressed protein 2 (Hand2). Normal uterine function, epithelial cell proliferation, apoptosis suppression, and regulation of lactoferrin and Muc1 production are all dependent on oestrogen. To promote implantation, a number of substances, including cytokines, growth factors, lipid mediators, homeobox transcription factors, and ion transporters, interact autocrine, paracrine, and juxtacrine ways (Li et al., 2011; Franco et al., 2012; Namiki et al., 2018). However, as spacing was affected when uterine muscle activity was disturbed by relaxing during early pregnancy in rats, (Pusey et al., 1980) proved that uterine myometrial activity plays a substantial role in embryonic spacing (Pusey et al., 1980). Furthermore, (Legrand et al., 1987) stated that uterine movement is regulated by noradrenergic transmission via myometrial post-synaptic 1-adrenoceptors for blastocyst distribution and spacing in the rat uterus (Legrand et al., 1987; Yoshinaga & genetics, 2013).

2.2.2 Mechanism of implantation

Implantation is a sequence of events where conceptus is positioned and then fixed within the uterus and then becomes more intimately related with maternal tissues, both morphologically and physiologically (Welsh & technique, 1993). Implantation takes place during a certain time period known as the 'implantation window,' during which

the endometrium becomes receptive to active trophoblast. The endometrium's receptivity is influenced by ovarian steroids. Pregnancy progressed through the creation of the placenta and organogenesis (Figure 2.3). It is a very intricate process that is mediated by different biomolecules in each phase. Changes in these events result in pregnancy loss. During implantation, complex proteins and polysaccharides of the endometrium degraded by Cathepsin-D and Alkaline phosphatase (ALP). Via the autolytic activity of epithelial cells and interaction with the maternal blood supply, cathepsin-D is linked to trophoblast invasion into the maternal endometrium. ALP is necessary for implantation, uterine receptivity, decidualization, and defence against bacterial endotoxin in a mouse model. Implantation failure is caused by changes in these molecular markers. As a result, in a letrozole-induced hyperandrogenic rodent model, it would be interesting to investigate implantation enzyme activity and steroid status in the endometrium during the implantation window. In other words, these biomarker changes serve as indicators of pregnant status (Solanki et al., 2018).

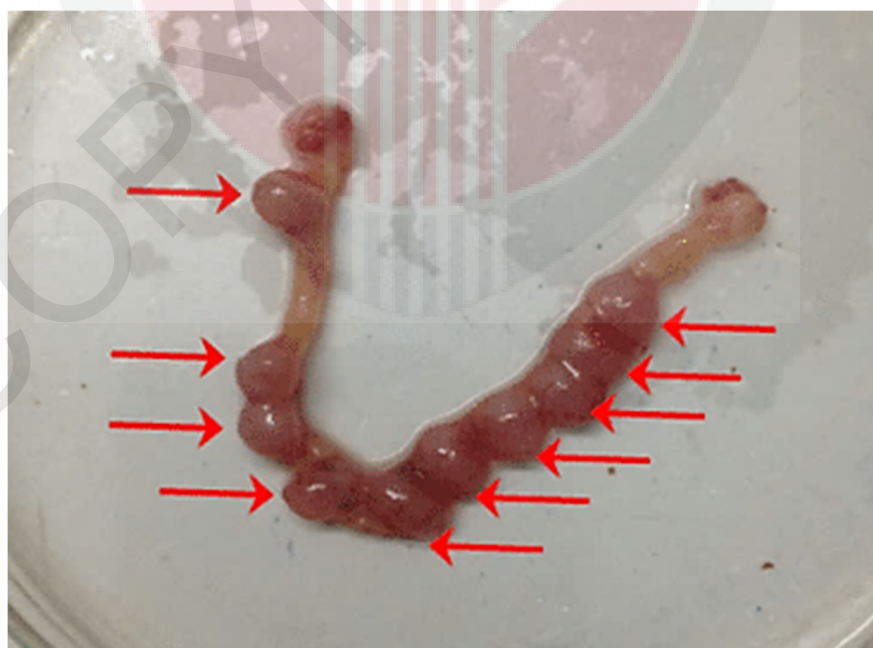


Figure 2.3: Gravid uterus from a rat showing implantation sites with embryo beads indicated by red arrows
(Solanki et al., 2018)

2.2.3 Implantation steroid hormones

The uterus, the ovaries, and the breasts are all influenced by the hormone's oestrogen and progesterone. The nuclear oestrogen receptor (E2R) and the progesterone receptor (P4R) are primarily responsible for the uterine effects of oestrogen and progesterone. Oestrogen activates the P4 gene, which enhances progesterone activity and is necessary for uterine epithelial growth (Albishtue et al., 2018). The endometrium's correct sensitization by ovarian steroid hormones, which take place during the reproductive cycle, is a requirement for a successful implantation. Before oestrogen can cause the endometrium to temporarily become sensitive to blastocysts, progesterone must prime the endometrium for 48 hours (Svalander et al., 1990; Geisert et al., 2019). LIF and IHH mediate oestrogen and P4 activity, respectively. Despite the complexity of mammalian oestrogen and progesterone (P4) signalling, embryonic implantation requires both (Figure 2.4).

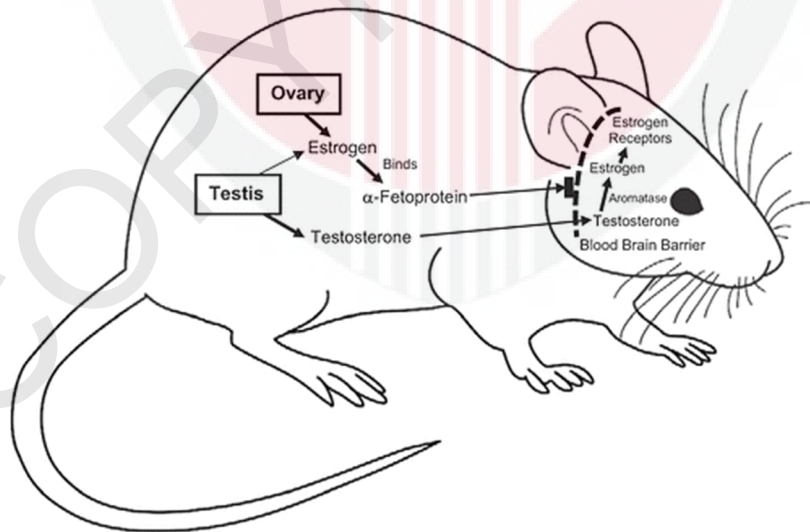


Figure 2.4: An illustration of postnatal male sexual behaviour is imprinted by plasma testosterone crossing the blood-brain barrier and turning into oestrogen in the brain. The female brain imprints female sexual behaviour as a result of oestrogen binding to fetoprotein, which blocks passage through the blood-brain barrier

(Geisert et al., 2019)

2.2.4 Epidermal growth factors (EGF)

The salivary and Brunner's glands generate a polypeptide with a molecular weight of 6,045 called epidermal growth factor (EGF). Nevertheless, hormones and growth factors are crucial components of this regulatory system since they are physiologically active peptides. Two growth-stimulating peptides, EGF and TGF are thought to be crucial in this process. It was found to be a heat-stable, nondialyzable polypeptide that was immune to pepsin, trypsin, and chymotrypsin and make up around 0.5 percent of the submaxillary gland's protein content. It is a 53 amino acid single-chain polypeptide that is deficient in lysine, phenylalanine, and alanine (Tarnawski et al., 1992; Dvorak et al., 2000). Furthermore, (Tarnawski et al., 1992) stated that EGF is a mitogenic peptide which is acid-stable. It has been shown to induce DNA synthesis in rodent gastric epithelium, stimulate cell proliferation with trophic effects, and speed up gastroduodenal ulcer healing (Tarnawski et al., 1992).

EGF appears to be produced as a high molecular weight compound in the submaxillary gland. According to sedimentation equilibrium, the molecular weight of this complex, which is made up of two EGF molecules with each having a molecular weight of 6,045 including two 29,300-dalton molecules of a binding protein, is around 74,000. The complex can dissociate into its component parts at pH values of less than 5 or greater than 8, and it will reassociate on its own at a pH of 7. However, reassociation of EGF with the binding protein requires the presence of a carboxy terminal (Arg-Leu) residue. The binding protein has a terminal arginine esteropeptidase. The fact that EGF appears to act as a tumour promoter is significant; it prevents density-dependent inhibition of cell growth in culture and enables cell development in a medium lacking serum (Hilaire & Jones, 1982; Bor et al., 2000).

Yet, a number of cell types can be stimulated to proliferate, differentiate, and migrate by EGF and its ligands. Proliferation and the development of cancer have been related with regards to the EGF family, and more specifically the heregulin receptors. Microvascular endothelial cells; kidney; lung; mammary gland; are just a few of the developmental systems that have been proven to be affected by these stimuli and to be induced to undergo development and morphogenesis. The postnatal testis expresses transforming growth factor α (TGF- α), a similar ligand, as well as EGF (Prigent & Lemoine, 1992; Levine et al., 2000). Moreover, the 6-kDa polypeptide hormone known as EGF has a potent mitogenic effect. However, other scientists' use of a sensitive immunoassay to identify EGF in the brain was unsuccessful, despite utilising three distinct anti-EGF antibodies to find EGF-like substances in the rat brain. While there are small amounts of EGF precursor mRNA in the brain, no physiological EGF targets have been identified. The "EGF domain" is found in several membrane-bound or secreted proteins. Plasminogen activator, tumour growth factor, and Caenorhabditis lin-12 and Drosophila notch products are examples. The latter two genes demonstrate that the EGF domain might function as a mediator of intercellular communication since they have an impact on cell lineage and neuroblast development (Gómez-Pinilla et al., 1988).

2.2.5 Vascular endothelial growth factors (VEGF)

In the years following its discovery, the angiogenic and permeability-inducing peptide family known as vascular permeability factor (VPF) and VEGF have been the subject of intense research. As was previously indicated, VEGF appears to play a crucial role in regulating endothelial cell activity in both normal and abnormal states. Cloning and

sequencing cDNA from human tissues and cells revealed five variants of VEGF that encode for peptides of 206, 189, 165, 145, and 121 amino acids, respectively. Bovine and rodent homologues for VEGF₁₈₈, VEGF₁₆₄, and VEGF₁₂₀, have been amply validated. Although the most common type, VEGF₁₆₄, is commonly mentioned, no system has directly quantified the relative abundance of the many VEGF mRNA variations. Several VEGF mRNA types were identified using PCR, but their relative abundance was not measured (Pierce et al., 1995).

In adults, the uterus and ovaries are the primary sites of angiogenesis (the formation of new blood vessels from preexisting vessels) during the gestational phase of the reproductive cycle. For implantation and placentation to take place successfully, angiogenesis and enhanced vascular permeability are necessary. VEGF has a major impact on vascular permeability and angiogenesis. The effects of oestrogen and progesterone are mediated by shifts in the spatial and temporal expression of VEGF and its receptors in the uterus. Despite this, several experimental investigations show that exogenous VEGF treatment can improve skin flap survival, hasten the maturation of flap prefabrication, and stabilise endothelial function in ischemic flaps (Ma et al., 2001; Matsumoto et al., 2002; Zhang et al., 2003).

Furthermore, it has been shown that VEGF plays a crucial role in the proliferation, migration, and tubule formation of vascular endothelial cells via its receptors, kinase insert domain-containing receptor/foetal liver kinase 1 (KDR/Flk-1) and fms-like tyrosine kinase 1 (Flt-1). Although VEGF is the primary angiogenic hormone involved in the regulation of sinusoidal endothelial cells (SECs), other angiogenic hormones such as platelet-derived growth factor and fibroblast growth factor also play a role.

Both hepatocytes and nonparenchymal cells produce VEGF mRNA during partial hepatectomy (PH), proving the importance of VEGF in liver regeneration (Taniguchi et al., 2001; Zhang et al., 2003). VEGF expression is boosted by hypoxia and is necessary for tumour-associated angiogenesis. VPF another name for VEGF, is a characteristic that may be helpful in vitreal haemorrhaging (Robbins et al., 1997).

2.2.6 Epidermal growth factors receptor (EGFR)

Four transmembrane receptors that are a part of the receptor tyrosine kinase (RTK) superfamily are collectively referred to as the EGFR family: ErbB1/HER-1, ErbB2/Neu/HER-2, ErbB3/HER-3, and ErbB4/HER-4 are all types of epidermal growth factor receptor (Sibilia et al., 2007). The EGFR family of receptors acts by ligand-dependent dimerization and activation of the cytoplasmic tyrosine kinase (Levine et al., 2000). Heparin-binding EGF, amphiregulin, epiregulin, b-cellulin, and transforming growth factor- α are all examples of EGFR ligands. ErbB receptors become activated when a ligand binds to them, homo- and heterodimers are formed, the intrinsic kinase domain is activated, and some tyrosine residues in the cytoplasmic tail are phosphorylated. The kind of the numerous signalling molecules attracted to the phosphorylated receptor is thought to control the selectivity of the cellular responses. The number of signalling pathways that can be triggered is increased as a result of this and ErbB receptors' capacity to form homo- and heterodimers. Key pathways activated downstream of ErbB receptors include the pro-survival and anti-apoptotic PI3K-Akt-mTOR cascade and the proliferation- and differentiation-regulating Ras-Raf-MEK-ERK1/2, STAT3, and STAT5 pathways (Hynes & Lane, 2005; Sibilia et al., 2007).

Also, the EGF domain's physiological targets host the EGF receptor (EGFR), a 171-kDa transmembrane glycoprotein with tyrosine kinase activity that appears to mediate EGF action. The cellular localization and developmental appearance of EGFR may shed light on the EGF domain's activity and target of action in the brain (Gómez-Pinilla et al., 1988). However, studies with EGFR knockout mice revealed that this receptor serves multiple roles crucial during embryonic and postnatal growth. To a certain extent determined by their genes, mutant mice experience slower growth and death at various stages of development. While EGFR mutant embryos die at 11.5 days of gestation in a 129/Sv context (MF1, C3H), these mice can live until delivery (C57BL/6) or postnatal day 20 in other contexts. Results from EGFR-deficient mice show that EGFR deficiency has the greatest effect on epithelial and glial cells, which are also the cell types where EGFR has been shown to be overexpressed in malignancies in humans (Sibilia et al., 2007).

2.2.7 Proliferating cell nuclear antigen (PCNA)

DNA polymerase delta's auxiliary protein has a molecular weight of 36k, it is required for DNA replication and goes by the acronym PCNA. The cell cycle's S phase is when this protein's synthesis peaks after beginning in the late G1 phase. Proliferating cell nuclear antigen (PCNA) is a ubiquitous, very stable protein, according to study. PCNA was found in both healthy and abnormally proliferating cells by researchers employing immunohistochemistry (IHC) to detect cell replication. However, it has been discovered that in paraffin-embedded tissues, IHC detection of replicating cells with PCNA is insensitive. Yet, a research by (Pierce et al., 1995), proved that by adopting an enhanced IHC approach on formalin-fixed, paraffin-embedded tissues, Increased

efficiency in antibody usage and a shorter primary incubation time without enzyme predigestion contributed to enhanced PCNA staining. Regular cell replication assessments will be made simpler with improved PCNA detection in formalin-fixed, paraffin-embedded tissues. The enhanced biotin-streptavidin amplified detection method allows for shorter primary incubation times without the need for enzyme predigestion, more effective antibody usage, and stronger staining of the PCNA antigen (Pierce et al., 1995).

2.2.8 Metallothioneine (MT)

Most living things include metallothioneins (MTs), a type of stress protein. By encapsulating the metal as complexes bound to cysteine, the MTs play a crucial part in the detoxification of heavy metals. Originally identified as a zinc (Zn)-binding protein that binds cadmium (Cd), MT is now widely distributed in a number of organs. MT is thought to have a number of roles in the regulation of Zn and copper (Cu) homeostasis, acute phase response, heavy metal detoxification, as well as functioning as an oxygen-free radical scavenger, due to its high sulfhydryl content (Takano et al., 2000; Kara et al., 2005; Mukhopadhyay et al., 2009). Metallothioneins (MT) are low-molecular-weight proteins (6-7 kDa), which are rich in cysteine and have a strong affinity for binding heavy metal ions in biological systems, such as Zn, Cd, Cu, silver (Ag), mercury (Hg), and others. A sensitive sign of exposure to heavy metals is the increase of MT production. According to numerous research, kidneys from different species can significantly increase their MT expression when exposed to cadmium. Although MT may help build nuclear inclusion bodies, which may protect against lead toxicity, there is no evidence that lead exposure causes MT to be induced in the kidney (Wang et al., 2009; Banni et al., 2010). Mammals have at least four primary MT

isoforms. MT-1 and MT-2 are the most abundantly expressed MT isoforms, while MT-3 and MT-4 are mostly localised to the brain and stratified squamous epithelia. A sensitive biomarker of exposure to heavy metals is the induction of MT synthesis (Banni et al., 2010). The most prevalent Metalloproteases come in two forms, metallothionein-1 (MT-1) and metallothioneins-2 (MT-2). MT is induced in most organs by a variety of stressors and chemicals, including metal ions, with MT-1 being the main isoform induced by metal ions (Ali et al., 2002).

Since primary antibodies cannot distinguish between the MT-I and MT-II isoforms, the two are typically handled as a single species (MT-I/II). MT-I/II is distributed throughout the rodent body in several organs. Multiple studies have demonstrated that MT-I/II expression increases in the brain following brain damage. It has been established that in MT-I/II^{+/+} mice, hepatic zinc concentration rises in response to upregulation of MT-I/II expression (Pankhurst et al., 2012). Transgenic mice deficient in both MT-I and MT-II (called MT-null mice) were recently created via gene targeting. According to numerous studies, paraquat, UV radiation, and acetaminophen can all produce oxidative stress in MT-null animals. In vitro tests on primary grown MT-null cells revealed that they were more vulnerable to the free radical-inducing agents paraquat and tert-Butyl hydroperoxide. It indicates that MT may work in vivo to shield tissues from free radical damage, regardless of how it works (Hidalgo et al., 1988; Takano et al., 2000).

2.3 Edible bird's nest (EBN)

Using the secretions from its salivary glands under the mouth, the swiftlet constructs a bird's nest. Nesting can be used to give canaries a secure place to roost and breed.

More than 24 species of swiftlets use echolocation to hunt insects. However, only two swiftlet species now create a marketable edible bird's nest (EBN). *Aerodramus fuciphagus* and *A. maximus* are two examples of species in the genus *Collocalia* under the family *Apodidae*. EBNs are nests constructed from retched saliva. The swiftlet's white, half-bowl-shaped nests are made from the mucin glycoproteins secreted by two specialised sublingual glands found beneath the tongue during reproduction (Figure 2.5). The soft, viscous nested cement (glutinous secretion/mucin glycoprotein) quickly dries and hardens when exposed to air. *A. fuciphagus* nests had fewer feathers and other contaminants than other birds. Most EBNs are solid cement that resembles a nest with feathers. Thus, processors and retailers like them. Southeast Asia produces most EBNs.

Swiftlets may leave their cave or home in the morning to seek for food. Swiftlets don't leave their nests because they don't want to meet other birds or animals. Swiftlets usually return to their nests before dusk after a successful forage. They only return to the swiftlet house or cave to lay eggs and sleep. Currently, the bulk of EBN goods are consumed both in these regions as well as in North America, with the majority of EBN raw materials coming from Asian nations like China, Malaysia, Indonesia, Vietnam, Thailand, and the Philippines. A lot of artificial bird houses have been built to conserve the natural habitat of swiftlet breeding populations while simultaneously increasing the quantity and quality of EBN. The farmhouse EBN, also referred to as the home EBN, is a nest built inside a swiftlet house that was manufactured by humans and has a structure that resembles a cave. Because of the prerecorded swiftlet calls, these birds choose to nest in the swiftlet house (Wu et al., 2010).

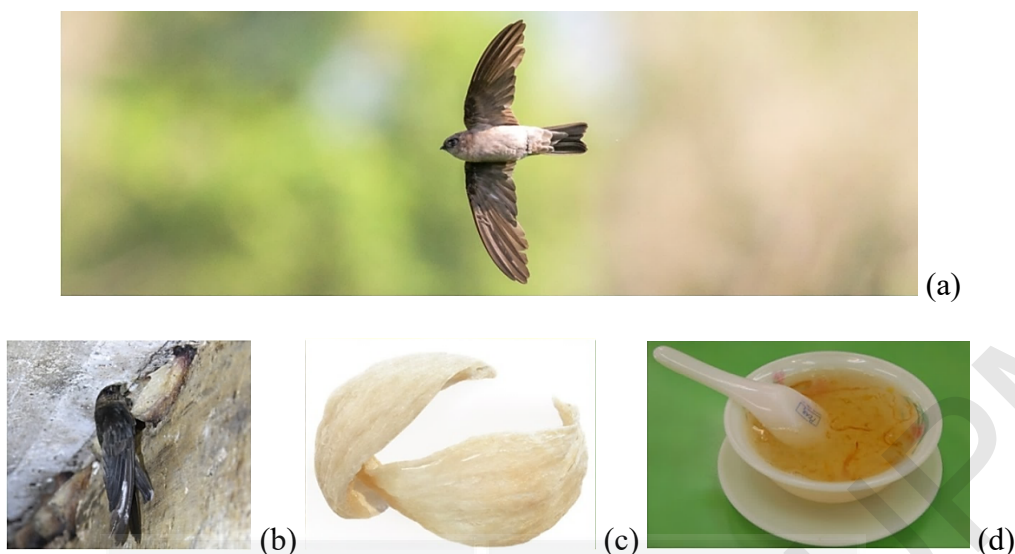


Figure 2.5: The pictures display (a) the swiftlet bird, (b) the swiftlet bird creating a nest, (c) the finished product, (d) the bowl of soup in which it is used
 (Source: <https://delishably.com/world-cuisine/Edible-Bird-Nests>)

Extensive research into EBN's tonic characteristics has revealed that it prompts the body to produce mitotic hormones and the growth factor for epidermal growth, which in turn leads to cell repair and activation of the immune system. Recently, a number of carbohydrate molecules, including novel compounds containing sialic acid and glycoconjugates, have been found in EBN (Guo et al., 2006). The therapeutic potential of EBN, including its capacity to treat osteoarthritis, prevent osteoporosis, and strengthen the immune system, has been scientifically shown. EBN administration has also been demonstrated to alleviate metabolic diseases, encourage wound healing, increase cognitive function in ovariectomized rats and increase the length and weight of uterine and vaginal tissues (Jaffar et al., 2021).

2.3.1 Biochemical Analysis of EBN

Asians have historically consumed EBN due to its nutritional properties. It is said to stimulate vitality, slow down ageing, and improve general wellbeing. Moreover,

research suggests that it possesses anti-inflammatory, antioxidant, and bone-strengthening qualities (Yida et al., 2015). Except some amino acids and fatty acids, carbohydrates and glycoproteins make up the bulk of EBN. EBN is considered to be a nutrient-rich food due to its composition. The Chinese prepare the delicacy known as bird's nest soup by cooking nest material with sugar in a double boiler. According to some estimates, ethnic Chinese in North America make up the second largest EBN user group in the world (after Hong Kong). Glycoproteins rich in amino acids, carbohydrates, calcium, salt, and potassium make up the majority of EBN. The majority of EBN's weight is made up of protein. According to (Marcone, 2005), Lipid 0.14–1.28%, ash 2.1%, carbohydrate 25.62–27.76%, and protein 62–63% make up the edible bird's nest. In the East, EBN is a delicacy on par with caviar in terms of prestige and price. EBN is a valuable source of water-soluble proteins, amino acids, carbohydrates, iron, inorganic salts, and fibres, among other nutrients, and has long been used as a health supplement in Traditional Chinese Medicine (TCM). EBN also helps heal malnutrition, boost the immune system, improve metabolism, improve the appearance of the skin, and lessen asthma (Marcone, 2005; Babji et al., 2015; Hun et al., 2015). Additionally, (Matsukawa et al., 2011) found that oral administration of EBN extracts increased skin thickness and calcium concentration in ovariectomized rats, as well as bone density and calcium concentration (Matsukawa et al., 2011).

The EBN has been used as an anti-aging, anti-cancer, and immune boosting hormone replacement prophylactic treatment in several illnesses. The biological properties of the EBN supplement, such as cellular proliferation, may be responsible for the observed protective effect. EBN is thought to be composed of testosterone, estradiol (E2), progesterone (P4), luteinizing hormone (LH), follicle stimulating hormone

(FSH), and prolactin. Sialic acid, which is found in EBN, has several molecular routes for cell proliferation that are controlled by the hormone E2. VEGF and IL-6, which inhibit embryonic neuron death by obstructing caspase three activation and so suppressing cell apoptosis, are also said to be present in EBN. Like epidermal growth factor (EGF), EBN promotes cell proliferation and survival. Therapeutic effects and regulation of anti-inflammatory and antioxidant genes by EBN have been demonstrated (Albishtue et al., 2020). EBN has been discovered to include hormone-like compounds in recent years, including mitogen and the avian epithelial growth factor. These compounds are thought to be the origin of EBN's reported rejuvenating qualities because they encourage cell division and proliferation, which can improve tissue growth and regeneration (Lin et al., 2009).

2.3.2 Hormonal compositions of EBN and its effect on reproduction

It has been proven via prior research that EBN contains the reproductive hormones testosterone, E2, P4, luteinizing hormone (LH), follicle stimulating hormone (FSH), and P. The regulation of the mammalian reproductive system is thought to be aided by these hormones, which are regarded as regulatory hormones. The uterus, along with the ovaries and the breasts, makes up the female reproductive system and contains the receptors for P4 and E2 activity. The high sialic acid (SA) content of EBN is another biological characteristic that has been suggested to have an impact on mammalian reproduction and fertility. SA, present in most mammalian cells, makes up most of EBN's sugar. Ovarian follicles get their nourishment from SA, a glycoprotein. The vitellin lipoglycoprotein, which is present in oocytes as yolk proteins, builds up in oocytes as the ovary matures (Albishtue et al., 2019). Another study, conducted by

(Ma & Liu, 2012), discovered that giving castrated male rats EBN boosted their serum levels of LH and testosterone. According to this research, EBN may be utilised to treat erectile dysfunction (Ma & Liu, 2012). Recent studies on EBN increased uterine proliferation and differentiation in rats subjected to lead acetate poisoning, increasing fertility and embryo implantation (Quddus et al., 2021). EBN enhances the differentiation and proliferation of ovarian tissues, as evidenced by increased steroid receptor expression, according to a separate study conducted on female rats (Albishtue et al., 2018). EBN preserves uterine glandular secretions such as growth factors, hormones, and transport proteins and aids conceptus development (Albishtue et al., 2018). Through an integrated mechanism of maintaining antioxidant-ROS balance and upregulating the genes for PCNA, VEGF, and EGF, oral supplementation of EBN at a dose of 60-120 mg/kg bwt has been shown to protect against and prevent changes in the histomorphology and function of the reproductive system brought on by Pb toxicity (Albishtue et al., 2019; Falah et al., 2019).

2.4 Reproductive toxicology

"Any chemical action that interferes with reproductive ability or capacity" is meant by reproductive toxicity, which also has an impact on nursing and the development of the offspring. A significant source of public concern is the vulnerability of reproductive and developmental outcomes to toxic substances. However, there is now a huge gap between the number of drugs used commercially and those that have had their effects on reproduction investigated. It is very difficult to model the full reproductive cycle in a single in vitro system because it is one of the most intricate biological processes. The answer to this conundrum is to break down this cycle into its biological elements,

which can then be researched separately or in tandem. This may result in a better understanding of the biological activity and underlying mechanism of each specific chemical (Nikolaidis, 2011; Younis et al., 2017). Developmental toxicity is described as "unfavourable effects caused during pregnancy or as a result of parental exposure" that "may manifest at any point in the organism's life span" by the Globally Harmonized System. Consequently, exogenous chemical exposure before conception, during pregnancy, or during postnatal development from infancy to sexual maturity might result in developmental toxicity. Due to spontaneous miscarriages, stillbirths, birth defects, early postnatal mortality, and reduced birth weight caused by developmental toxicity, the developing organism develops structural abnormalities, changes in growth patterns, functional insufficiencies, and eventually dies. To quantify the hazards of exogenous chemicals on human health, however, animal models are used. Testing on animals is performed to identify compounds that might harm human fertility. Animal studies on reproductive toxicity examine each stage of the reproductive cycle individually (reproduction/fertility, prenatal development, and postnatal development) or as part of a procedure (two-generation test). In the area of developmental and reproductive toxicity, (Nikolaidis, 2011; Younis et al., 2017) assert that animal testing is the sole technique to determine whether a drug may have an adverse effect on human reproduction (Nikolaidis, 2011; Younis et al., 2017).

People and animals are harmed by the bioaccumulation of toxic metals in water and animal feed. They pose a risk to human health since they can impair kidney function and cause issues with the heart and neurological system (Volkov & Ezhkova, 2020). Lead, arsenic, mercury, and cadmium are examples of toxic metals that can harm animal reproductive. Animal biology is influenced by chromium, copper, iron,

selenium, nickel, zinc, manganese, molybdenum, and cobalt (Guvvala et al., 2020). The toxicity of the substance is dose and time dependent. The ovarian granulosa cells are damaged by toxic metal accumulation in the follicular fluid of the female reproductive system, which reduces hormone synthesis and lowers the quality of the oocytes. Moreover, toxic metals can result in pregnancy loss or early calving. They may also pass through the placenta and interfere with the foetus's growth (Akarsu et al., 2017; Akar et al., 2018; Verma et al., 2018; Das et al., 2021). Yet, multiple studies have discovered that exposure to dangerous metals is connected to disturbances in gametogenesis (Keil et al., 2011; Guvvala et al., 2020; Kabala et al., 2020; Volkov & Ezhkova, 2020).

Additionally, environmental pollutants can have a serious effect on fertility as well as reproductive health. After one year of consistent, unprotected sex or donor insemination, infertility is said to have set in. For women over the age of 35 or those with extra infertility risk factors, this period is shortened to six months. According to data from nationwide surveys conducted between 2015 and 2017, 13.1% of American women between the ages of 15 and 49 have experience with infertility (Capotosto & Jurgens, 2020). Infertility can have many different reasons, including ovulatory dysfunction, low ovarian reserve, tubal factor, male factor, multifactorial aetiologies, and unexplained infertility (Jain & Singh, 2021). Exogenous compounds known as endocrine disrupting chemicals (EDCs) can cause both male and female infertility when they are exposed, especially during pregnancy or puberty. These variables can worsen obesity, diabetes, and endocrine cancers, which impact fertility (Birnbbaum). EDCs include flame retardants, pesticides, lubricants, phytoestrogens in soy products, bisphenol A [BPA] products, phthalates, tea, and lavender oils in the home, and

diethylstilbestrol [DES] and oestradiol in pharmaceuticals. EDCs have been linked to female ovarian dysfunction, infertility, and PCOS (Diamanti-Kandarakis et al., 2009; Gore et al., 2015; Troisi et al., 2016). Heavy metal exposure has also been connected to infertility. Like lead from paint or mercury and arsenic from food and water, exposure can happen in the natural environment as well as in the home or workplace. Lead, mercury, cadmium, and arsenic are heavy metals that are known to harm reproductive function. Lead paints, water from lead pipes, cosmetics, building sites and herbal supplements are among the hazards and can be consumed that contain lead (Gore et al., 2015; Stel & Legler, 2015; Jain & Singh, 2021). A study conducted by (Gerhard et al., 1998) revealed a correlation of gynaecological diseases (uterine fibroids, miscarriages, hormonal disorders), with several heavy metals and clinical factors (age, body mass index, nationality). This study suggested that the DMPS test, which involves administering the chelating chemical 2,3-dimercaptopropane-1-sulfonic acid, was a helpful and additional diagnostic technique in such circumstances (Gerhard et al., 1998).

2.4.1 Indomethacin (IMC) toxicity

In recent years, NSAIDs have seen the highest usage rates. Over thirty million people use this group of medications on a daily basis to relieve pain and inflammation. Anti-inflammatory NSAID indomethacin alleviated swelling produced by the indolic hormones serotonin and tryptophan. It was first prescribed in 1963 for the treatment of rheumatoid arthritis, ankylosing spondylitis, gout, degenerative joint illnesses, acute musculoskeletal problems, post-operative swelling and inflammation, and discomfort associated with primary dysmenorrhea. It has anti-inflammatory properties in addition

to analgesic ones, and there is proof of both central and peripheral activity. The cyclooxygenases (COX) 1 and 2 and polymorphonuclear leucocyte motility are both inhibited by indomethacin. Indomethacin is no longer routinely used for analgesic or antipyretic therapy due to the high prevalence and severity of side effects associated with long-term use. Because there is currently little human consumption or commercial demand for the chemical, it is very affordable and widely available in most third-world countries, particularly Nigeria. Reports of its use as a rodenticide in most homes and farms have increased in the meantime. Due to the increased risk of toxicosis in humans and animals in connection with the potential consumption of indomethacin-killed rodents (Olusegun Taiwo & Lawal Conteh, 2008).

Indomethacin is a frequently used anti-inflammatory and analgesic drug. As a result, it was used to treat the inflammation, swelling, stiffness, and joint discomfort associated with rheumatoid arthritis, degenerative joint disorders, ankylosing spondylitis, gout, and acute musculoskeletal difficulties. Indomethacin may elevate the likelihood of developing major systemic toxicity without warning signals, especially when administered over an extended period of time. It promotes lipid peroxidation, which can lead to the creation of free radicals, which are particularly toxic to tissue and cause oxidative tissue damage. Current research has discovered liver damage, nephrotoxicity, and intestinal toxicity depending on the amount and duration of use (Elshama et al., 2014). When more than 5 mg/kg BW of indomethacin was provided, stomach ulceration the drug's most severe side effect occurred within 2 weeks. The most frequent adverse effects, except those on the GI tract, were renal and liver damage. Chronic toxicity experiments at dose levels ranging from 2 to 5 mg/kg/day found renal amyloidosis and papillary necrosis in mice, as well as cellular infiltration

of the kidney and liver in rats. The results of the carcinogenicity investigation in rats and mice (dose levels: 0.5, 1.0, and 2.0 mg/kg/day; later dropped to 1.5 due to high mortality) showed no tumorigenic effect. It has been shown that the majority of NSAIDs prevent PG synthesis by obstructing COX1 and COX2 enzymatic activity. Lethargy, abdominal swelling, and decreased appetite were among the aberrant behaviours noted during the experiment and they continue to be signs of IMC-induced toxicity (Kaplan et al., 2012).

2.4.2 IMC effect on female reproductive system

Reproductive toxicity experiments in rats and mice (dosages of 0.5, 1, 2, and 4 mg/kg/day) did not reveal any teratogenicity. Delays in foetal ossification led to smaller birth weights. Maternal toxicity increased foetal loss (resorptions) and abnormalities were seen at higher dosages (5-15 mg/kg/day) in mice. When given to rats for the final three days prior to parturition, indomethacin delayed parturition and increased neuronal necrosis in the brain (diencephalon) of newborn rats. When newborn rats were given the same dose during their first three days of life, this impact was not noticeable (Kaplan et al., 2012). After providing indomethacin, a general COX inhibitor, to pregnant Wistar rats, researchers assessed reproductive performance, liver morphology, and postmortem features. Oral indomethacin was given to study participants (n=10) on days 3 and 4. From placebo to 8.40 mg/kg/day (Damasceno et al., 2008). At therapeutic levels of 0.32 and 1.68 mg/kg body weight (bwt), indomethacin in humans did not cause embryotoxicity or death. High doses of indomethacin (8.40 mg/kg) prevented significant development in certain foetuses by interfering with the implantation process. Peritonitis was discovered in the animals

administered 8.4 mg/kg of antibiotic after necropsy and during bacteriological testing. It was believed to be the cause of these species' demise (Damasceno et al., 2008). However, other studies have shown that indomethacin reduced the number of implantation sites, induced severe gastrointestinal haemorrhage, and killed animals in pregnant rats when administered at doses of 3 mg/kg twice day, 5 mg/kg daily, and 10 mg/kg daily (Poyser & Acids, 1999). At 10 mg/kg/day, indomethacin significantly enhanced both preimplantation and post-implantation losses (Sookvanichsilp & Pulbutr, 2002).

2.4.3 Lead acetate (PbA) toxicity

Toxic metals such as lead, cadmium, mercury, and arsenic are present in our environment. There are numerous polluted sources from which we absorb these metals. These heavy metals can build up in different body parts, such as the reproductive organs, where they may have an impact on physiology and structure. There are, however, a number of defence mechanisms to mitigate the impacts of these heavy metals. One method for preventing the negative effects of heavy metals is the production of stress proteins (Mukhopadhyay et al., 2009).

Pb is a dangerous heavy metal that is not necessary and is abundantly present in the environment. Pb is ingested by people through their food, drink, air, and soil. It is also typical in places of employment like smelters and industries that produce lead. A number of organ systems, including the cardiovascular, renal, immune, and reproductive systems, have been proven to be hazardous after exposure to Pb. Despite improvements in public health measures and large decreases in adult blood Pb levels,

Pb exposure remains a major health risk worldwide. Pb is a toxin that affects practically all organ systems and is especially harmful to the central nervous system. The nervous system is particularly vulnerable to Pb poisoning at low doses, and the developing brain is more at risk than the adult brain. Many factors, such as oxidative stress, mitochondrial dysfunction, and excitotoxicity, contribute to Pb-induced neurotoxicity. By generating oxidative damage to vital macromolecules like lipids, proteins, and DNA, oxidative stress can harm the brain. Animals exposed to Pb have been reported to produce more ROS, modify their immune systems, and have disruptions in the pro/antioxidant balance in developing rat brains. Low blood lead levels also limit ALA-D, which produces heme and is sensitive to lead. Suppressing ALA-D, which produces reactive oxygen species, is a good sign of lead toxicity (Ahamed & Siddiqui, 2007; Kim et al., 2011; Lalith Kumar, 2014).

Lead disturbs cellular metabolism and is harmful by attracting strong oxidizers (O_2) and active metals (sodium, potassium). This interference generates reactive oxygen species (ROS) like superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^\cdot$), and lipid peroxides, which damage proteins, membrane lipids, and nucleic acids. One of the proposed mechanisms for lead toxicity is that lead-induced oxidative stress disrupts the prooxidant/antioxidant balance in mammalian cells, which then adds to the deleterious effects. The body is shielded against reactive oxygen species' damaging effects by the antioxidant enzymes that are released (ROS) (Kumar et al., 2018). The growth of ROS too much and exposure to xenobiotic metals like lead during development have also been connected to neurological disorders like Parkinson's and Alzheimer's. A typical cell has a delicate equilibrium of prooxidants and antioxidants; if this balance shifts in favour of prooxidants, the condition is known

as "oxidative stress." If the stress is severe or ongoing, significant cell damage may result. Catalase and superoxide dismutase are antioxidant enzymes that help to maintain homeostasis for proper cell function (Bolin et al., 2006; Bokara et al., 2008) (Figure 2.6).

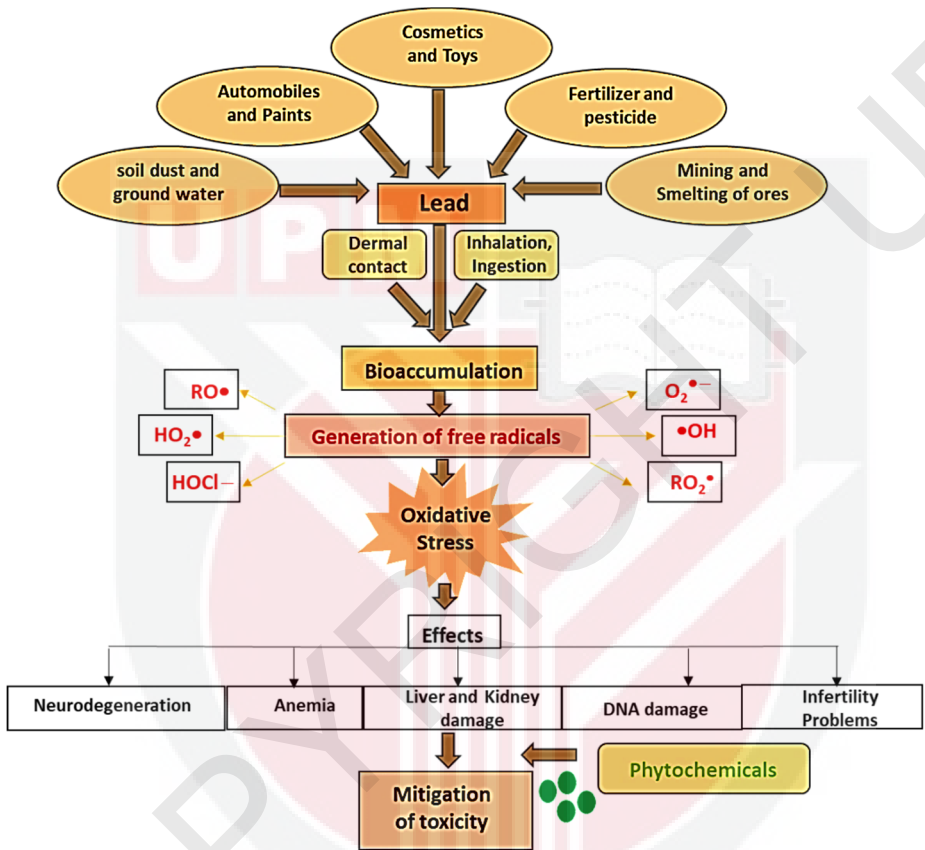


Figure 2.6: Mitigation of lead toxicity process
(Kumar et al., 2018)

2.4.3.1 Lead acetate effect on the female reproductive system

Animal infertility and associated production losses, as well as malnutrition and ovulatory or hormonal disorders, are thought to be significantly influenced by lead. There is, however, a dearth of information on how environmental poisons affect breeding and how they affect female reproduction. The uterine gland secretions that

control ovulation, follicular growth, and menstruation have all been demonstrated to be inhibited by long-term lead exposure in monkeys. Also, it can delay the pubertal rat's vaginal opening and reduce the frequency of mice's ova implants and pregnancies. Recent research has shown that therapeutic meals with antioxidant activity can shield physiotherapists from the damaging effects of heavy metals (Albishtue et al., 2019). Even though lead is known to have adverse effects on female reproduction. There has not been much research on the connection between lead and infertility, though. Lee et al. compared the levels of lead and cadmium in the blood of infertile and pregnant women to see if there is a connection between self-reported infertility and these metals among American women (Lee et al., 2020). Even after controlling for potential confounders, low blood lead levels were associated with self-reported infertility (geometric mean = 0.50 g/dL; odds ratio (OR) for lead per two-fold rise in blood metal levels = 2.60; 95% confidence interval) (Lee et al., 2020). Lead also plays a role in cow infertility caused by induction. Lead accumulation in cow alveolar fluid severely reduces folliculogenesis. Folliculogenesis was suppressed and the number of primary follicles that were atrophic after 15 weeks of lead infusions in mice at 10 mg/kg. Male sperm density, sperm count, sperm quality, and sperm fructose were all diminished by Leydig cell atrophy. Fructose changed azoospermia, asthenozoospermia, and morphological abnormalities in sperm by decreasing succinate dehydrogenase and alkaline phosphatase concentrations (Keil et al., 2011; Tvrdá et al., 2013; Aglan et al., 2020; Kabala et al., 2020; Massányi et al., 2020).

2.4.3.2 Effects of Lead acetate on kidney

It has been demonstrated that lead can affect several organs, including the kidney. Lead promotes kidney oxidative injury by raising lipid peroxidation (LIP) (Ponce-

Canchihuamán et al., 2010). It has been proposed that lead-induced liver and kidney damage is primarily brought about by lipid peroxidation and oxidative stress. Lead reduced the activity of glutathione peroxidase, catalase, superoxide dismutase, and glutathione reductase in rats (El-Ashmawy et al., 2005). Lead exposure throughout pregnancy and after birth is harmful and has toxicological consequences on young male albino rats' kidneys. Degenerative changes like enlarged renal corpuscles, thickened glomerular basement membranes, and extensive tubular lining cell vacuolation were seen after exposure to lead (Nashwa A & Samira M, 2010). Some of the absorbed Pb is excreted in urine after being conjugated in the liver, but the rest accumulates in various organs where it interferes with molecular, cellular, and intercellular processes and leads to morphological changes that can persist long after Pb levels have dropped (Haouas et al., 2014).

2.4.3.3 Effects of lead acetate on liver

Lead in the liver is dangerous. Heavy metal poisoning requires the liver to store, biotransform, and detoxify substances. Histological investigation can detect liver somatic cell morphological changes caused by absorbed lead, which is deposited in soft tissues via the portal vein. The liver tissue of Pb-exposed people has the largest Pb storage among soft tissues, accounting for 33% of the total, followed by the renal cortex and medulla. Lead has been linked to higher levels of liver enzymes as well as histological liver damage and disturbances of normal metabolic processes. Lead poisoning leads to DNA damage via oxidative stress and ROS production in the liver. DNA can be damaged in two ways: by activating caspase 3 or by oxidative stress, which generates ROS like superoxide, hydrogen peroxide, hydroxyl radicals, and lipid peroxidation (Abdou & Newairy, 2006; Geraldine & Venkatesh, 2009; Haouas et al.,

2014). According to (Aziz et al., 2012), Pb was discovered to play a role in rat liver damage brought on by lipopolysaccharide (LPS), as well as oxidative stress brought on by nitric oxide (NO), and the production of tumour necrosis factor alpha (TNF-) (Aziz et al., 2012).

Haouas et al. found that lead acetate inhibits rat bwt growth. Lead acetate's direct toxicity, including gastrointestinal system toxicity and protein synthesis inhibition, reduces bwt, liver weight, and food intake. It is well known that liver AST and ALT values can indicate lead hepatotoxicity (Haouas et al., 2014). Acute hepatic necrosis is so sensitively indicated by ALT and AST activity. Lead has long been recognised to increase lipid peroxidation (LP) in the liver, which results in oxidative damage. A radical chain reaction caused by LP damages membrane integrity by oxidising cell components or inducing oxidative stress (El-Nekeety et al., 2009; Haouas et al., 2014). Lead causes liver damage due to lipid peroxidation (LIP). Both the end products of lead poisoning (LIP), such as hydroperoxides, singlet oxygen, and hydrogen peroxide, and the direct loss of antioxidant reserves contribute to free radical damage (Ponce-Canchihuamán et al., 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 Ethical approval

All research was conducted in accordance with the rules provided by the Institutional Animal Care and Use Committee of Universiti Putra Malaysia (UPM) (Project authorisation number: UPM/IAUC/AUR09/2016), which are displayed in (Appendix A).

3.2 Preparation of edible bird's nest

EBN produced by saliva of species of swiftlet bird (*Aerodramus fuciphagus*), was purchased from Mobile Harvesters (M) Sdn Bhd (House Bird's Nest; batch no: 1802030650), and it was kept between 25 and 32°C. The EBN sample conforms to the Malaysian Standards – Edible Birds Nest (EBN) - Specification (MS2334:2011). The EBN nests were prepared according to Chinese customs and collected from local EBN providers. After a thorough cleaning in water, the EBN nests were inspected for any contaminants. Thoroughly cleaned, the nests were allowed to naturally dry at room temperature before being crushed in an automated mixer. The EBN powder was kept at 4°C and the rats were given weight-based doses while the nests were still at room temperature (BUCHI.400, Switzerland). The EBN solution was created by dissolving 1 g in 100 ml of distilled water and heating it for 45 minutes at 56°C (Albishtue et al., 2018). The conventional technique of consuming EBN includes heating it with purified

water after it has been softened by soaking in water for several hours (Lv et al., 2023). The EBN solution was then administered to the rats at ambient temperature.

3.3 Nutritional composition of EBN

The EBN sample conforms to the Malaysian Standards – Edible-Birdnest (EBN) - Specification (MS2334:2011). Briefly, the sample contains 62.8% protein, 14.9% moisture and 10.1% sialic acid. The nitrite content was found to be 17.4 ppm, well below the tolerance level of 30 ppm. The microbiology standards (total plate count, coliforms, *E. Coli*, *Salmonella* sp., *Staphylococcus aureus*, yeast, and mould) and heavy metals (Pb, Cd, As, and Hg) also comply with the MS2334:2011 standard. The calories and carbohydrate content of several foods were analysed in recommendations for nutrition labelling and claims. To find the sialic acid, High-Performance Liquid Chromatography (HPLC) was employed. The nutritional breakdown of EBN is displayed in (Appendix B).

3.4 Preparation of lead acetate and indomethacin solution

From Oxford LabCorp. in India, lead acetate was purchased. With its chemical formula $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$, and 325.29 g/mol is its molecular weight (CAS: 6080-56-4). For eight weeks, oral gavage of a 1% (w/v) PbA solution diluted in distilled water was administered to rats at a weight of 10 mg/kg (Albishtue et al., 2018). The study made use of a commercially available 25 mg IMC (Methacid) tablet that was kept between 25 and 30°C (Sunward Pharma Company, Malaysia). IMC powder 1g was dissolved in one hundred millilitres of distilled water to create an IMC stock solution. These solutions were then cooled to 4°C. To create a model of an embryo implantation

problem, 4.33 mg/kg of IMC was administered between days three and four of pregnancy.

3.5 Animals and experimental design

After seven days of acclimation, the 24 mature female SD rats (6x4 groups) in each group of the IMC experiment were purchased from the Centre of Animal Resources, Faculty of Veterinary Medicine, Universiti Putra Malaysia, and their body weight ranged from 130 to 150 g. EBN supplementation was given orally once a day. Group 1 (control group) was given distilled water, which is devoid of any minerals, salts, or other materials that could change the outcome of the experiment (Zolfalizadeh et al., 2023). For the remaining groups IMC was used with the following doses: Groups 2 (4.33 mg/kg bwt), Groups 3 (4.66 mg/kg bwt), and Groups 4 (5.00 mg/kg bwt). The aim of this study was to develop a model of developmental defects that impede proper implantation. On the other hand, 30 SD rats were split up into (6x5 groups) for the (second experiment), Group 1 (Negative Control) received distilled water; Group 2 (Positive Control) received IMC (4.33 mg/kg bwt); Group 3 (G3) received IMC+EBN (4.33 mg/kg+60 mg/kg bwt); Group 4 (G4) received IMC+EBN (4.33 mg/kg+90 mg/kg bwt); and Group 5 (G5) received IMC+EBN (4.33 mg/kg+120 mg/kg bwt). The doses of EBN were chosen after doing exploratory research at a level of IMC (4.33 mg/kg bwt), as detailed out in Chapter 4. Both IMC and EBN were given once daily in the morning for a period of 8 weeks. Every week during the course of the treatment, the bwt of each group of rats was determined and recorded. Pregnant rats were given ketamine (30 mg/kg bwt) and xylazine (10 mg/kg bwt) followed by blood collection for hormone analysis on the eighth day of pregnancy.

Adult female SD rats forty-eight at a body weight of (130-150 g) were collected and given a seven-day acclimatisation period. The animals were fed a conventional diet of fat, protein, and sugars while being kept at a consistent temperature and humidity (Animal Feed Gold Coin Brand, Malaysia). The Institutional Animal Care, Faculty of Veterinary Medicine, Committee of Universiti Putra Malaysia's guidelines were followed, and the animals received humane treatment. After a settling period, the rats were split into five groups, each containing six animals, and vaginal cytology was used to verify the regularity of oestrous cycles: The negative control (Group 1) was given distil water; the positive control in Group 2 was provided with PbA 10 mg/kg bwt; Groups 3 and 6 received PbA 10 mg/kg bwt plus graded concentrations of 60, 90, and 120 mg/kg bwt of EBN and 20, 40, and 60 mg/kg bwt of hydrolysed EBN, respectively. All animals received oral doses of PbA and EBN once daily until the conclusion of the eighth week. The present investigation utilised the same PbA dosages as (Albishtue et al., 2019). EBN has been described to be safe and optimum effect at the dosage values of 30, 60, and 120 mg/kg bwt. However, dosage values of 60, 90, and 120 mg/kg bwt for the EBN were considered in current study as the precedent by (Albishtue et al., 2019). Throughout the course of the treatment, the bwt of the rats in each group was assessed and noted on a weekly basis. Oestrous rats were weighed and sacrificed after eight weeks of exposure and a heart puncture was performed while the rats were under general anaesthesia to collect blood for hormone analysis. Weekly measurements and records of the rat's body weight were made for every group during the administration period. 10 mg of xylazine and 30 mg of ketamine/kg bwt were used to achieve general anaesthesia (Albishtue et al., 2019). The reproductive system was weighed using an automatic weighing balance after

being free of all connective tissue, and kidney and liver tissues were also collected.

The gross exams revealed any evident irregularities.

Experimental design:

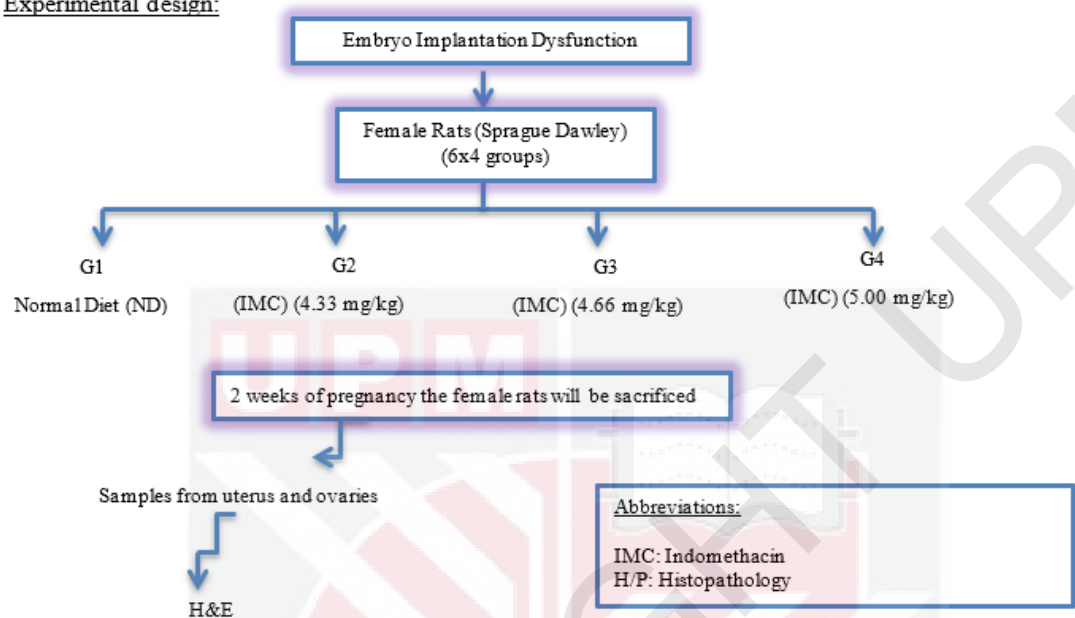


Figure 3.1: Experimental design for exposure to IMC

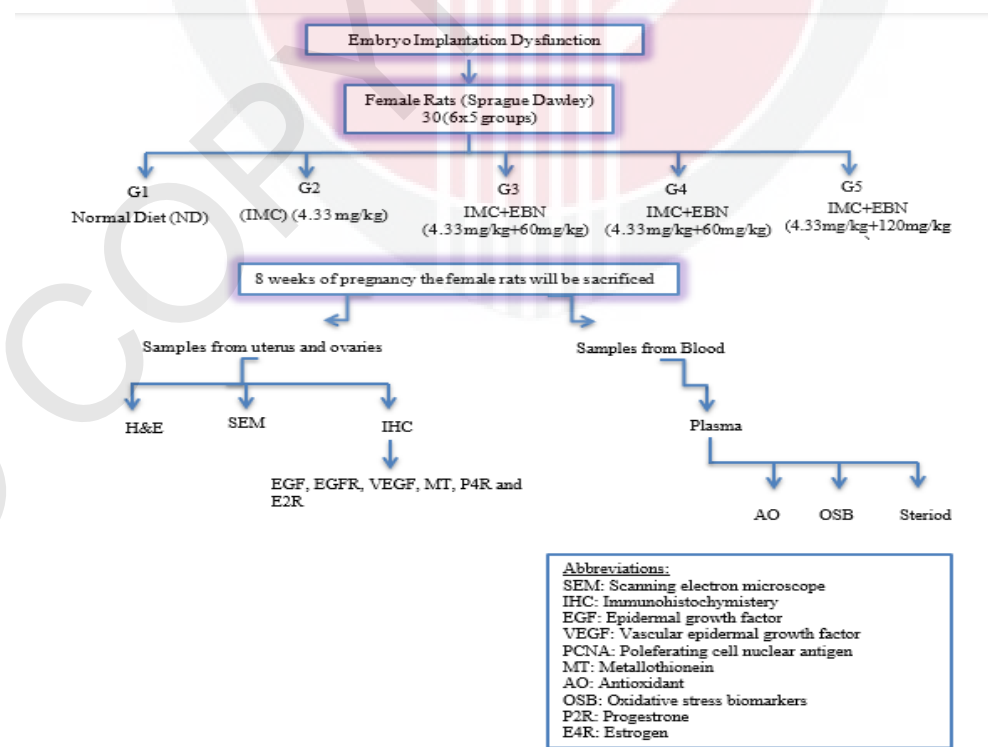


Figure 3.2: Experimental design for exposure to IMC and EBN

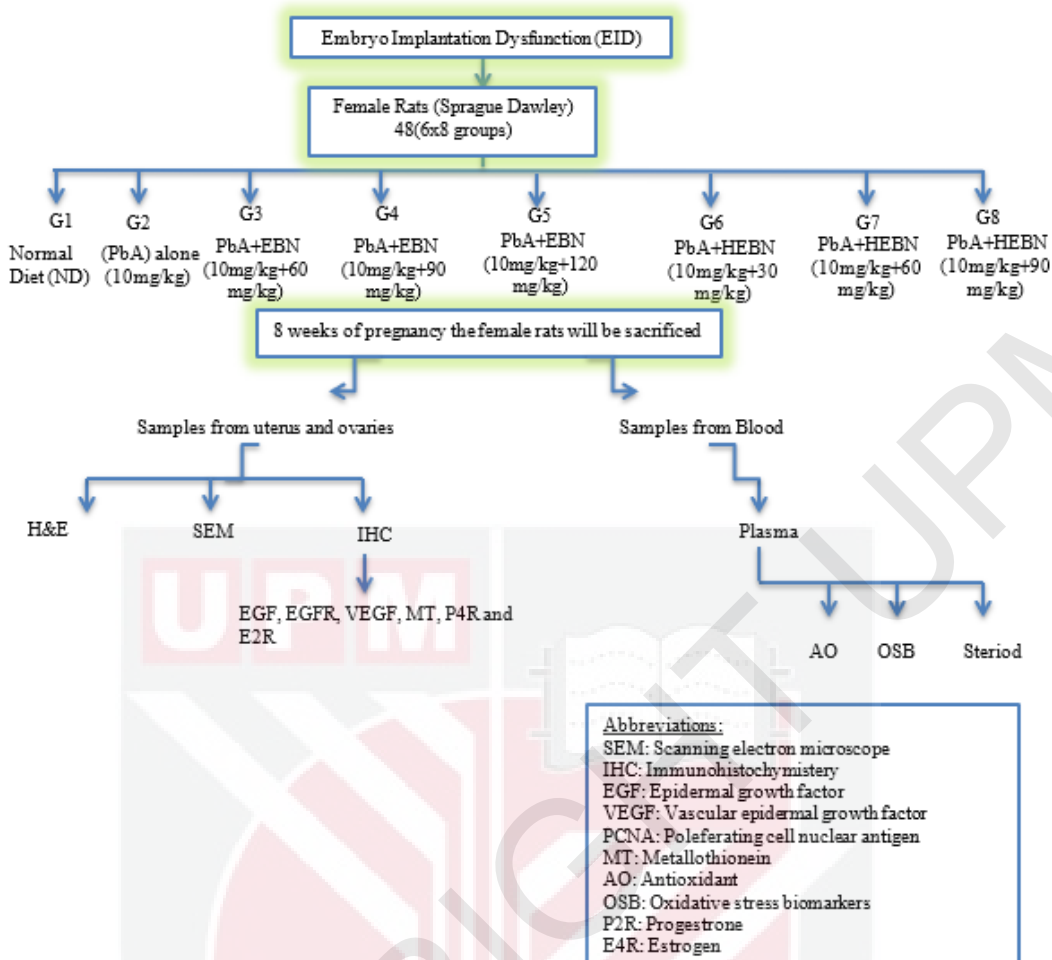


Figure 3.3: Experimental design for exposure to PbA toxicity and EBN

3.6 Determination of oestrous cycle phases and synchronization

Two doses of 0.5 μ of cloprostenol were given intraperitoneally to the rats three days apart to synchronise their circadian rhythms before the start of the experiment (Pallares and Gonzalez, 2009). Every day of the experiment, between the hours of 9:00 and 10:00 a.m., Cotton-tipped wooden swab sticks were used for vaginal smears. The points were gingerly inserted into the vagina to a depth of approximately 1.0 cm after being moistened by submerging them in distilled water. Rotating the swab's tip allowed it to be spread across a clean microscope slide (75 mm 25 mm). After methanol treatment and staining for three minutes with 5% Giemsa stain, the slide was

examined under a microscope (Leitz Labourlux-S, Wetzlar, Germany) to evaluate the percentages of various cell types in the vaginal smear and the stage of the cycle, use 40× objective lenses. (McLean et al., 2012).

3.7 Macroscopic and microscopic Examination of ovary and uterine tissues

Ovarian tissue samples of euthanized rats were collected after the rats were anaesthetized and slaughtered, cleaned with sterile saline, weighed, and measured in length, then preserved for 12 hours in 10% neutral buffered formalin shown in (Figure 3.4) were conducted at Histopathology laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia. The preserved tissues were then divided into 4µ tissue slices using a microtome (Leica 2235 Microtome, USA), placed on a glass slide, and stained with haematoxylin and eosin (H and E) stain. An ovarian morphometric study was performed in accordance with (Romeu et al., 2011), with a few modifications. The middle portion and the periphery were split into sections. We measured the thickness of the surface epithelium, counted ovarian follicles, and counted interstitial cells using medical image analysis software (Motic Imaging Version 2.0, China). The form of the cells, the number of layers encasing the oocyte, and the presence or absence of antral space were used to categorise follicles into the following types. There exist four separate types of follicles: the primordial, primary, secondary, and antral follicles (Luo et al., 2012). A degenerating oocyte or a fractured oocyte nucleus, as well as disordered granulosa cells, were seen in atretic follicles (Juliani et al., 2008), in each region were counted using medical image analysis software (Appendix F).

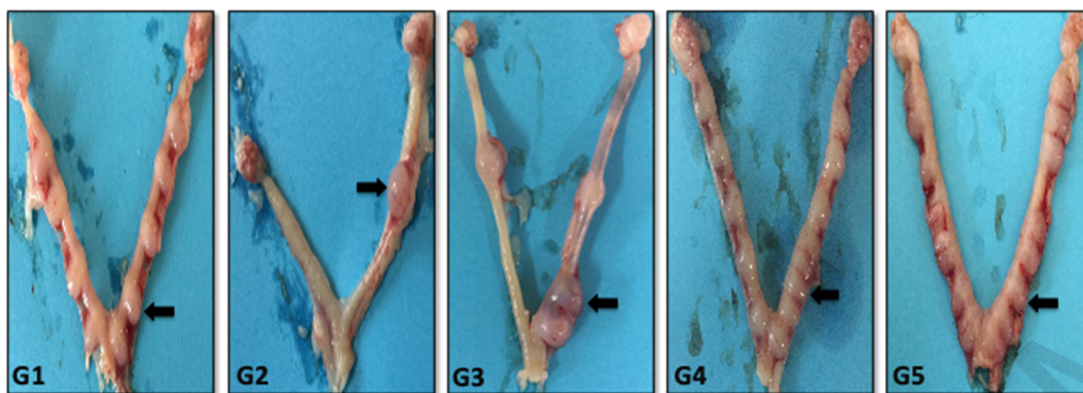


Figure 3.4: Uterus and ovary tissue samples. Black arrows indicate the small beads (embryos)

3.8 Macroscopic and microscopic examinations of the uterus, kidney and liver tissues

The uterus of the euthanized rats was taken out, cleaned in normal saline, weighed, and measured before being sacrificed. All the experiments were conducted at Histopathology laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia. The gross inspection highlighted any immediately noticeable irregularity that was identified. The tissue samples were divided into sections and stained with (H&E stain) as previously mentioned after being fixed in 10% formalin for 12 hours. Changes in the endothelial lining and the quantity of uterine glands were examined under a microscope in the samples (Muhammad et al., 2013). Sections were taken from the middle region to the periphery. Image Analysis programme (Motic Image plus 2.0, China) measured uterine gland number, LE thickness, and GE thickness. The liver's left lateral side and right medial lobes were cut transversely, while the kidney's left and right lobes were cut longitudinally.

3.9 Histopathological lesion examination

A 10% formalin solution was used right away to preserve the uterus. Paraffin blocks were made from the tissue. For histological analysis of tissues (H&E) stain was used. Using a medical imaging microscope with a camera (Motic Image plus 2.0, China), the number of follicles, interstitial cells, and adaptive cellular alterations was counted along with the number of uterine glands, luminal epithelium (LE), glandular epithelium (GE), and other histological modifications. Furthermore, representative photomicrographs were captured. To determine the histopathological score for lesions, an improved version of the methodology applied by (Othman et al., 2016). Six microscopic regions on three slides from each organ were examined for scoring at various magnifications (200x and 400x). On a scale of 0 to 3, inflammatory cell infiltration, vacuolation, and loss of glandular and luminal epithelial integrity, and congestion were evaluated for the treatment groups and the negative control. On a scale of 0 to 3, the differences between the treatment and control groups were rated (Table 3.1).

Table 3.1: Scoring method for lesions

S/No:	Scoring	Percentage of lesions (%)
1	0	no lesions
2	1	less than 30% of the lesions are seen
3	2	less than 60% of the lesions are seen
4	3	more than 60% of the lesions are seen

3.10 Measurement of ovarian or uterine weight: body weight ratios and fertility index

The ovarian or uterine weight ratios and fertility index were calculated using the formulas as:

$$\text{ovarian/uterine body weight ratio} = \frac{\text{ovarian or uterine weight (g)}}{\text{body weight (g)}} \times 100$$

$$\text{Fertility Index} = \frac{\text{number of pregnant females}}{\text{number of animals copulated}} \times 100$$

3.11 Analyses of the expressions of EGF, EGFR, PCNA, VEGF, ER, and PR in ovarian and uterine tissues by immunohistochemistry (IHC)

Using immunohistochemistry, the expression of the protein was examined. Slices of tissue were cut into 4µm pieces, deparaffinized, and dried. For the antibodies against VEGF, ER, and PR, antigens were removed from the specimens by incubating them at 80°C for 10 minutes in 0.01 M citrate buffer (pH 6.0), and then letting them cool naturally to room temperature. Additionally, the antibodies EGF and EGFR were recovered by incubating at 800°C for approximately 10 minutes at a pH of 9.0, PCNA by incubating at 800°C for 20 minutes at a pH of 6.0 of which were followed by cooling at room temperature and pressure. The samples were rinsed for 1 minute in Tris-buffered saline (TBS), and then tagged using a Dako pen (manufactured in Glostrup, Denmark). To get rid of the extra wash buffer, tapping was utilised. The endogenous peroxidase was deactivated using a kit designed for use in animal research (Code K3954, Dako, North America). The specimen was covered in 0.03 percent hydrogen peroxide with sodium azide and then heated to 37 degrees Celsius for 10 minutes before being washed in TBS. In this study, the following rabbit antibodies were used: anti-E2R (1:200, Anti-oestrogen receptor alpha antibody [E115]-cHip, grade ab32063), anti-P4R (1:100, Anti-progesterone receptor [SP2], ab16661), anti-EGF (1:100, Ab77851), and anti-EGFR (1:500, Ab52894). After being rinsed in TBS, the samples were incubated with a biotin-labelled secondary antibody (Envision. System-HRP) for 40 minutes at 37 °C. Polymer Anti-Rabbit (Code K4002; Dako,

USA) or Mouse Anti-PCNA Antibody (PC-10; Code M0879; Dako, Glostrup, Denmark) was used, followed by washing, incubation with the mouse anti-PCNA antibody for 20 minutes, and finally detection with the labelled secondary antibody. Chromogenic staining was performed by washing the sample sections, mixing the DAB (3, 30-diaminobenzidine) stain for 5 minutes, and rinsing with distilled water. Following haematoxylin counter staining, slices were examined. A good reaction was defined as a yellowish-brown staining colour detected under a microscope (magnification 400x), with the density of the staining. Based on ten example fields, the approximate percentage of immunopositive cells was examined. The strength of the immunostaining was graded as follows: weak (grade 1), mild (grade 2), moderate (grade 3), strong (grade 4) or negative (0) (Panzan et al., 2013) (Table 3.2).

The total index was calculated using the following formula

=

[percentage of positive cells]

x

[immunostaining intensity]

Table 3.2: Scoring method used for evaluation of immunostaining intensity

S/No:	Scoring	The percentage of positive epithelial cells
1	Grade 0	no stained cells
2	Grade 1	1–25% stained cells
3	Grade 2	26–50% stained cells
4	Grade 3	51–75% stained cells
5	Grade 4	76–100% stained cells

3.12 Scanning electronic microscopic (SEM) examination of ovaries and uterus

The uterine tissue was divided into 1cm³ parts in order to prepare it for SEM. Fixation in 4% glutaraldehyde at 4°C for 16-24 hours was followed by three 10-minute soaks in 0.1 M sodium cacodylate buffer. There were four 10-minute acetone dehydration treatments (35%, 50%, 75%, and 90%) and three 15-minute 100% acetone changes.

For another half an hour, the samples were dried using liquid carbon dioxide in a critical point dryer (Baltec CPD, Germany). The tissue was then sputter coated with 12 nm gold palladium (SCD005, German) and double-sided taped onto aluminium stubs. The material was inspected using a Philips XL 30ESEM SEM (Holland) operating at 15 keV.

3.13 Blood plasma collection

Following the end of the treatment period, vaginal cytology was used to identify each rat's oestrous cycle stage. All of the animals in each group were killed on day eight of pregnancy. The SD rats were immediately put to sleep, and 1-2 ml of blood from their hearts were drawn into EDTA tubes. The blood was centrifuged at a velocity of 2500 rpm for ten minutes at a temperature of 4°C to separate the plasma (made in Kubota, Japan).

3.14 Assessment of plasma concentrations of progesterone (P4) and oestrogen (E2)

The experiments were conducted at Theriogenology and cytogenetics laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia. A commercially available enzyme-linked immunosorbent assay ELISA kit (E-EL-0090, Elabscience, USA) was used to assess the plasma levels of P4; the technique was carried out in accordance with the instructions provided by the manufacturer. Plasma levels of E2 were assessed using a commercial ELISA kit (ER1505, Fine test, China) (Appendix E).

3.15 Oxidative stress biomarker and antioxidant assay

The experiments were conducted at Theriogenology and cytogenetics laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia. Plasma SOD activity was evaluated using the Assay Kit for SOD (E.BC. K021, Elabscience, USA). Twenty litres of plasma, twenty litres of enzyme working solution, and two hundred litres of substrate application solution were used in the reaction. In a mixture, incubate at 37°C for 25 minutes. Absorbance at 450 nm was determined using microplate readers. Biomarker of oxidative stress; TBARS. By testing TBARS, the concentrations of lipid peroxides (LPO) are determined. Using a commercial kit, TBARS was measured to assess the peroxidation amount of lipid, in the plasma (E-1369Ra, Bioassay technology Labs, China).

3.16 Analysis of the expressions of Metallothionein in the uterus and ovary tissues by Immunohistochemistry

Immunohistochemistry (IHC) was used to examine the expression of metallothionein. Tissue sections (4 µm) were collected from the paraffin-embedded chunks and placed on the new glass slides, where they were let 15 hours to dry by air. A series of xylene and alcohol solutions were used to deparaffinize the slides. The sections were submerged in diluted target retrieval solution (pH 6.0) for a predetermined period of time to retrieve the antigen (Dako, North America). After being heated on high power for 10 minutes, the container containing the retrieval solution, and the submerged slides was allowed to cool naturally to room temperature. After discarding the Target Retrieval Solution (TBS), the sections were washed three times in tris buffered solution. The tissue was marked with a Dako pen after the excess wash buffer was

tapped off (Dako, Denmark). An IHC detection kit (Code ab236469, Abcam USA) was used to heat-inactivate endogenous peroxidase in the sample for 10 minutes at 37 °C. Samples were TBS-washed. To prevent endogenous immunoglobulins from reacting with the secondary antibody, the sample was incubated with the protein block at 37 °C for 10 minutes in TBS. After that, samples were incubated at 4 °C for 30 minutes with the rabbit polyclonal anti-Metallothionein antibody ab192385, diluted 1:150, from Abcam, USA. Lacking the primary antibodies, negative controls were created. TBS was used to clean the samples before the Peroxidase-labelled polymer secondary antibody, IHC detection kit was applied for 15 minutes at 37°C (Code ab236469, Abcam USA). The slides were developed using the chromogen DAB for 10 minutes (3, 30-diaminobenzidine). Rinse the stain with clean water to get rid of it. I counterstained the slides by submerging them in a haematoxylin wash for three minutes. Based on the degree of the immunostaining, the expression of metallothionein in different LE and GE cells was graded and divided into four categories: 0 denotes absence, A score of 1 indicates a weak positive effect, 2 a moderate one, and 3 a substantial one. Each sample included five parts and five fields chosen at random. We counted and ranked 100 LE cells and 100 GE cells from each sample. The following equation was used to determine the immunohistochemistry score. The Medical Image Analysis microscope (Motic Image plus 2.0, China) was used to examine an additional five slices and five fields from each sample (Albishtue et al., 2019).

3.17 Statistical analysis

The results were produced using Graph Pad Prism 6.0 (Graph Pad Software, San Diego, California) for the steroid hormone, OSB, and AO test results. Kruskal-Wallis

(a non-parametric test) was used to compare histology, histopathology, and immunohistochemistry. To compare UBWR, OBWR, SOD levels, and MT gene expression, one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-hoc test was performed. All bwt ratios were examined using a two-way analysis of variance (ANOVA) along with Bonferroni's multiple comparison testing and Tukey's post-hoc test. P-values less than 0.05 were used to signify statistical significance.



CHAPTER 4

EFFECT OF INDOMETHACIN (IMC) ON EMBRYO IMPLANTATION AND HISTOMORPHOLOGY OF UTERUS AND OVARY OF RAT

4.1 Introduction

The implantation of embryos depends on both molecular and cellular activities, in addition to the necessary implantation processes. These include preimplantation embryo development, blastocysts' escape from zona pelucida, and uterus differentiation to the receptive state (Andrabi & Maxwell, 2007; Lü et al., 2019). During ovulation and implantation, PGs such as PGE2 and PGF2 alpha play a very important role. The PGs formed by the action of COX are extremely important in order to ascertain a successful implantation (Salleh *et al.*, 2014). Nevertheless, the formation of PGs is restrained by NSAIDs. IMC remains an NSAID, analgesic, and antipyretic drug. IMC is administered to mammals subcutaneously, intravenously, or orally as a suppository and topical gel (Olivera-Muzante et al., 2019; Ma et al., 2020).

IMC is reported to be quite useful in veterinary medicine for treating inflammatory conditions. Rodents acquire a receptive uterus upon mating (Horton et al., 1992; Thompson et al., 2020) and have been seen to have a relatively short estrous cycle (4-5 days) (Lin et al., 2004; Sato et al., 2016). Their uterine endometrium is comprised of columnar epithelium cells and stromal cells (Cavalieri et al., 1998). However, after the IMC is injected into the animal, the COX enzyme is inhibited by NSAIDs, which normally subsists COX (1-2) are two notable isoforms. In the uterine luminal as well as the glandular epithelial cells a bigger expression of the COX-1 was observed

throughout morning of Pd4 (Poyser & Acids, 1999), but its expression in epithelial cells drops to nearly undetectable levels during the attachment reaction. During the attachment reaction, COX-2, on the other hand, is solely expressed at the blastocyst attachment site and is required for implantation in the luminal epithelium (Sato et al., 2016; Thompson et al., 2020).

The aim of the present study was to examine the use of IMC to determine embryo implantation failure in SD rats by evaluating embryo development and histomorphological abnormalities in the tissues of the uterus, ovaries, liver, and kidneys. On the other hand, not much study has been done on how well IMC therapy works to induce EID in SD rats. The purpose of this experiment was to create an EID model rat.

4.2 Methods

4.2.1 Experimental design

Each group contained cages housing a total of 24 mature female Sprague-Dawley rats that were about 12 weeks old (6 x 4, with 6 animals/group). Rats underwent an in vivo supplementation with IMC in accordance with Table 4.1 below after a 7-day acclimatisation period. The trial was carried out over the course of a 2-week period. IMC dose selection was done in accordance with a prior report by (Ismail et al., 2014). Histological studies of uterine, ovary, liver, and kidney tissues were carried out as presented in detail in Chapter 3.

Table 4.1: Group allotment of the animals and feeding regime in the experimental design

Group	Assignment (6 rats/group)	Type of feed (dose)/2 weeks
Control	G1	Normal diet +Normal saline
Treated Groups	G2	Normal diet +IMC (4.33mg/kg bwt.)
	G3	Normal diet +IMC (4.66mg/kg bwt.)
	G4	Normal diet +IMC (5.00mg/kg bwt.)

ND=Normal diet, IMC=Indomethacin, bwt=Body weight.

4.2.2 Oestrous synchronization and ascertaining of oestrous cycle phases

Oestrous synchronization and assessment of oestrous cycle phases (Figure 4.1 & 4.2) were performed as described in Chapter 3.

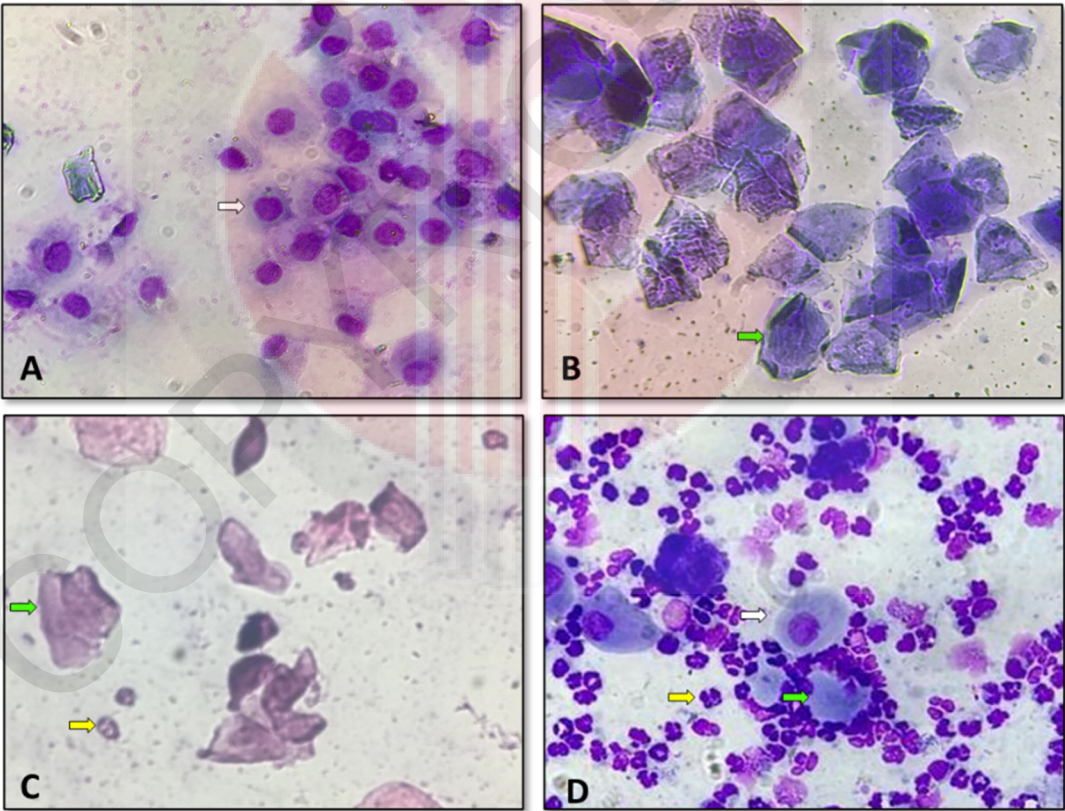


Figure 4.1: Vaginal cytology of the SD rats showing various stage of the estrous cycle

Where, (A) Proestrus, (B) Oestrus, (C) Metestrus, and (D) Dioestrus. Note: The nucleated epithelial cells are represented by the white arrows, the green arrows represent the cornified squamous epithelial cells, and the yellow arrows for the leukocytes. Giemsa stain, magnification 400x.

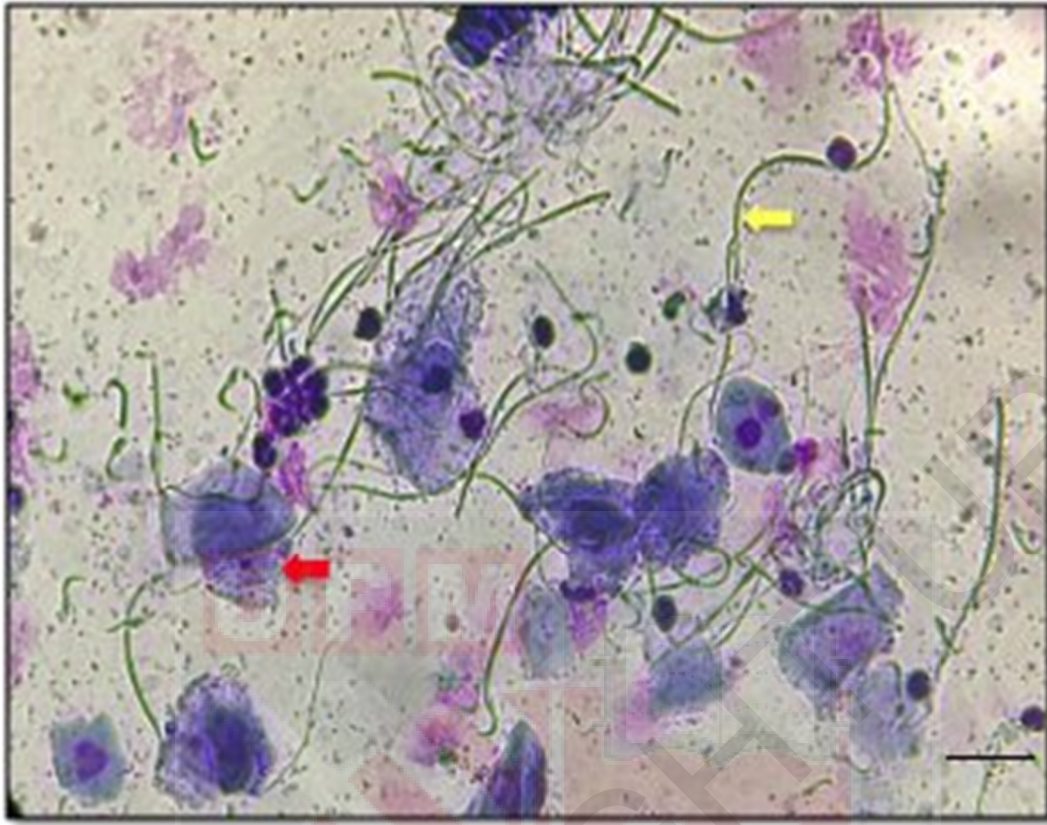


Figure 4.2: A micrograph of the vaginal smear at Pd1

Here, represented by the yellow arrow, are the large number of sperm, whereas the red arrows depict the cornified cells. Giemsa stain. 100µm scale bar

4.2.3 Statistical analysis

The luminal endometrial epithelium, follicle, and corpus lutea thicknesses were evaluated using Motic Images Version 2.0 Software (China). All data are presented using M SEM. One-way ANOVA with Dunnett's and Tukey's multiple comparison post-hoc tests were used to compare uterine and ovarian bwt gain and uterine luminal epithelium thickness, and two-way ANOVA with Bonferroni's multiple comparison tests were used to compare bwt, ovarian follicles, and corpora lutea. There was statistical significance at the p-value of 0.05. On a scale of 0–3, any variations between the treatment groups and the control were rated. On a 0-3, the differences between the treated and control groups were rated. Score 0 = no lesions, 1 = less than 30% of the

lesions are seen, 2 = less than 60% of the lesions are seen, and 3 = more than 60% of the lesions are seen. In order to compare histopathology scoring, a non-parametric statistical test (Kruskal-Wallis) followed by post hoc Mann Whitney U test for multiple comparison, was used. The significance level was less than 0.05. The pathologist assessing the sections was uninformed of the treatment that the rats were receiving (Albishtue et al., 2019). The rat treatment was concealed from the pathologist who examined the sections.

4.3 Results

4.3.1 Effects of IMC on maternal bwt, OBWR, and UBRW

The average bwt of rats was determined until the eighth day of pregnancy, as illustrated in Figure 4.3. All of the treatment groups, G2 (4.33 mg/kg bwt.), G3 (4.66 mg/kg bwt.), and G4 (5.00 mg/kg bwt.) showed a significant weight loss from Pd4, while G1 (Control) showed a weight gain after giving birth. The mean bwts of the groups of IMCS at 4.33 mg/kg bwt, 4.66 mg/kg bwt, and 5.00 mg/kg bwt were nearly identical, hovering around 260 g. Furthermore, the 4.33 mg/kg bwt group had a somewhat lower mean bwt of 245.667 g. The control group G1 (Normal diet and Normal saline) showed a noticeable advance in bwt, a significant decrease was observed in the bwt on Pd4 in the treated groups. On Pd8, the treatment group developed embryo implantation dysfunction in all cases.

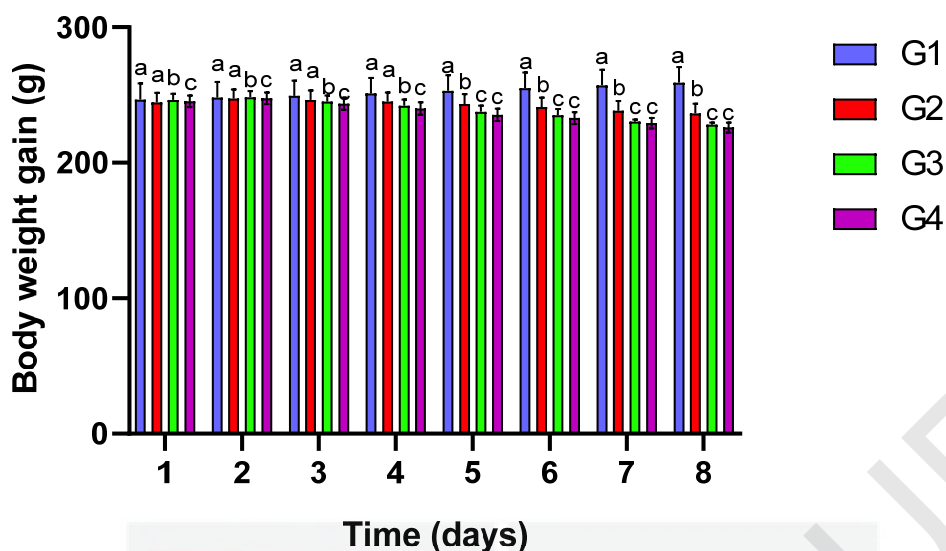


Figure 4.3: Effects of IMC administration on body weight gain after pregnancy

The data is expressed as Mean±SEM. The error bars showing different letters a, b and c within rows indicate significant difference at $p<0.05$. *G1=Control, G2= 4.33 mg/kg bwt, G3= 4.66 mg/kg bwt, G4= 5.00 mg/kg bwt.

Additionally, a statistically significant difference in OBWR was noted across the groups, with the highest OBWR ($p<0.05$) in the control group and the lowest in the 5.00 mg/kg bwt of the IMC group, as shown in Figure 4.4(A). Meanwhile, UBWR was found to be lower in 4.66 mg/kg bwt and 5.00 mg/kg bwt of the IMC group than in the 4.33 mg/kg bwt of the IMC group, whereas the control group had the greatest value ($p<0.05$), as illustrated in Figure 4.4(B).

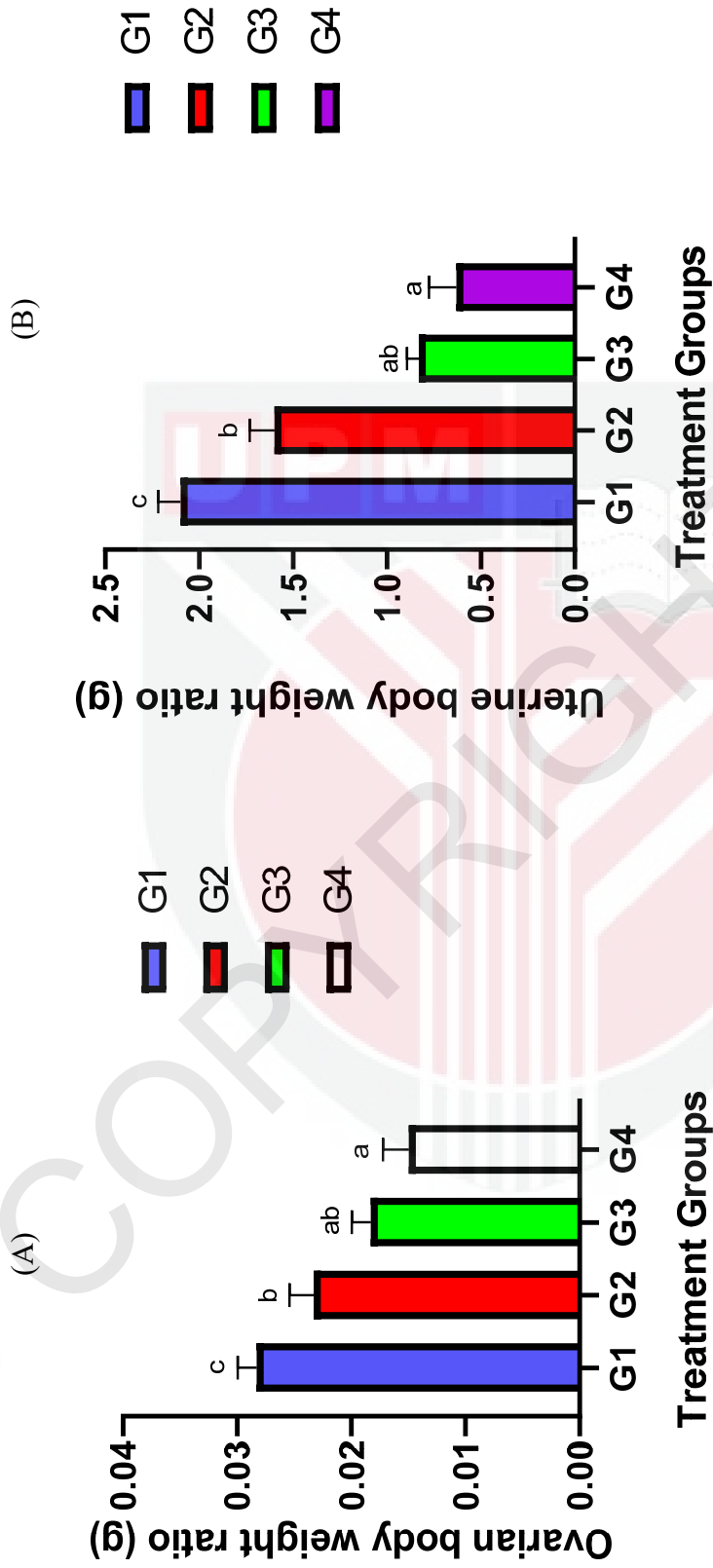


Figure 4.4: Effect of IMC on OBWR (A) and UBWR (B). The data are expressed as Mean $\pm$ SEM. The error bars showing different letters a, b and c within rows denotes significant difference at $p < 0.05$. *G1=Control, G2= 4.33 mg/kg bwt, G3= 4.66 mg/kg bwt, G4= 5.00 mg/kg bwt.

4.3.2 Embryo implantation rate and Fertility index

On the Pd8, a comparison of the control and treated groups was performed using the fertility index and the number of EIR. As illustrated in Figure 4.5, the sites were shown as little beads inside the groupings control and 4.33 mg/kg bwt of the IMC group. However, there were no implantation sites visible for groups at 4.66 mg/kg bwt and 5.00 mg/kg bwt of the IMC group. Thus, IMC treatment drastically decreased the number of implantation sites.

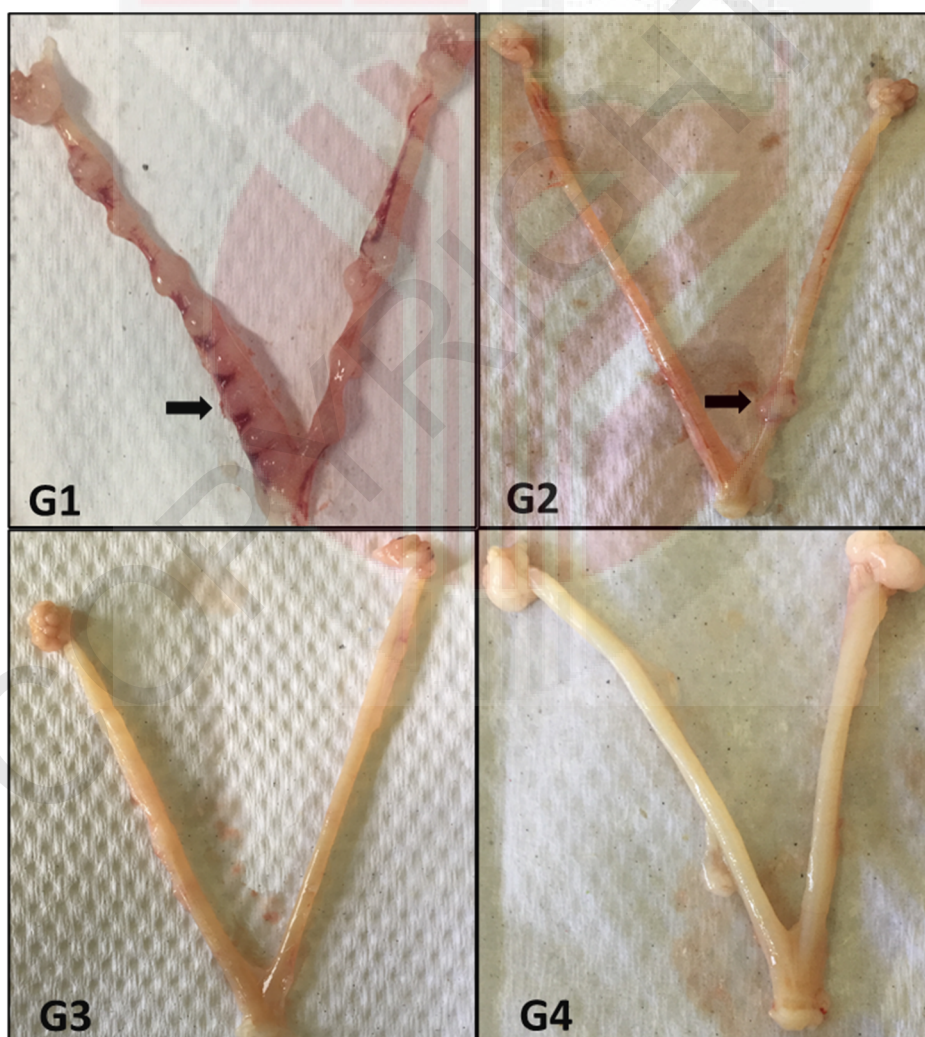


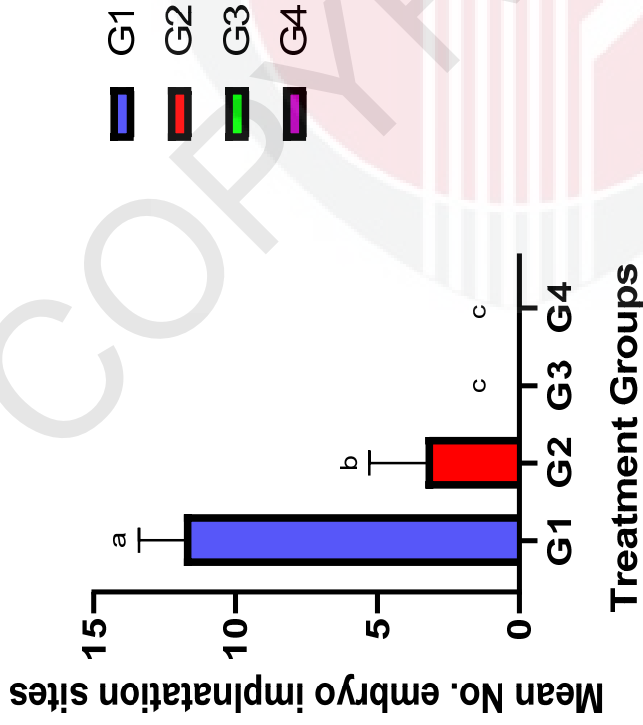
Figure 4.5: Implanted blastocysts in the uteri of the rats (shown with a black arrow)

*G1=Control, G2= 4.33 mg/kg bwt, G3= 4.66 mg/kg bwt, G4= 5.00 mg/kg bwt.

The effect of IMC on the EIR and fertility index is depicted in Figure 4.6. While the control group and 4.33 mg/kg bwt of the IMC group had significantly higher fertility index and EIR values as compared to other groups, both parameters decreased to zero for the groups 4.66 mg/kg bwt and 5.00 mg/kg bwt. The control group, which was not subjected to IMC, had the highest EIR, as seen in Figure 4.6(A). The effect of the IMC on the fertility index of all SD rats studied, shown in Figure 4.6(B).



(A)



(B)

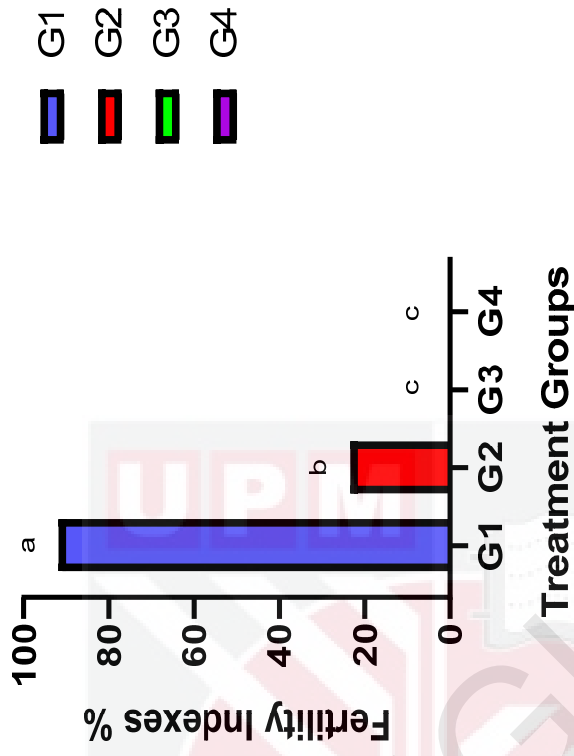


Figure 4.6: Effects of IMC on EIR (A) and fertility index (B). The data are expressed as Mean±SEM

The error bars showing different letters a, b, c, denotes significant difference at $p < 0.05$. *G1=Control, G2=4.33 mg/kg bwt, G3= 4.66 mg/kg bwt, G4= 5.00 mg/kg bwt. *Fertility index was calculated according to (Albistue et al., 2018) number of pregnant females/number of females with confirmed mating x 100.

4.3.3 Effects of IMC on the development of follicles, corpora lutea, and ovarian histomorphology

Figure 4.7 illustrates the histopathological results pertaining to the effects of IMC on the ovaries. No obvious pathogenic abnormalities were found within the ovaries. In terms of ovarian follicular dynamics, 4.33 mg/kg bwt and 4.66 mg/kg bwt of the IMC groups had a reduced ($p < 0.05$) dissemination of surviving follicles, including primordial, primary, and secondary follicles, as well as corpus lutea, when compared to the control group. As illustrated in Figure 4.8, the 5.00 mg/kg bwt group treated with the highest IMC dose had a greater proportion of atretic follicles than the other groups. As illustrated in Figure 4.8, the delivery of the IMC to the treatment groups resulted in a significant number of surviving follicles and corpus lutea. Also, histological sections of the ovaries of all treated groups except the control group showed changes like vasoconstriction and inflammation, as shown in Figure 4.9.

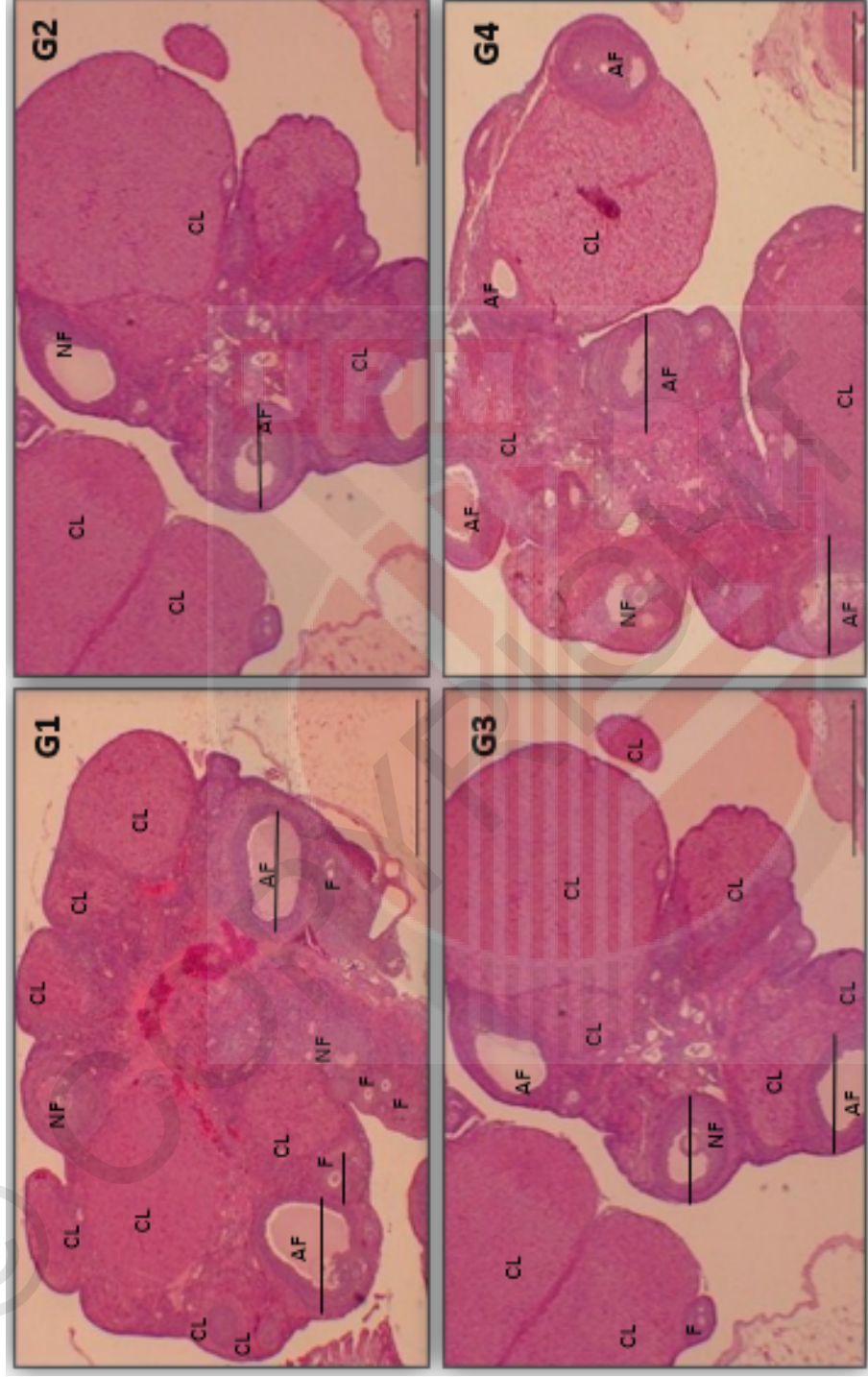


Figure 4.7: Photomicrographs of control and IMC exposed ovaries

Note: Follicular unit = F; antral follicle = NF; atretic follicles = AF; corpus luteum = CL. H&E stain. Magnification 400x. The line represents the diameter of the follicles.

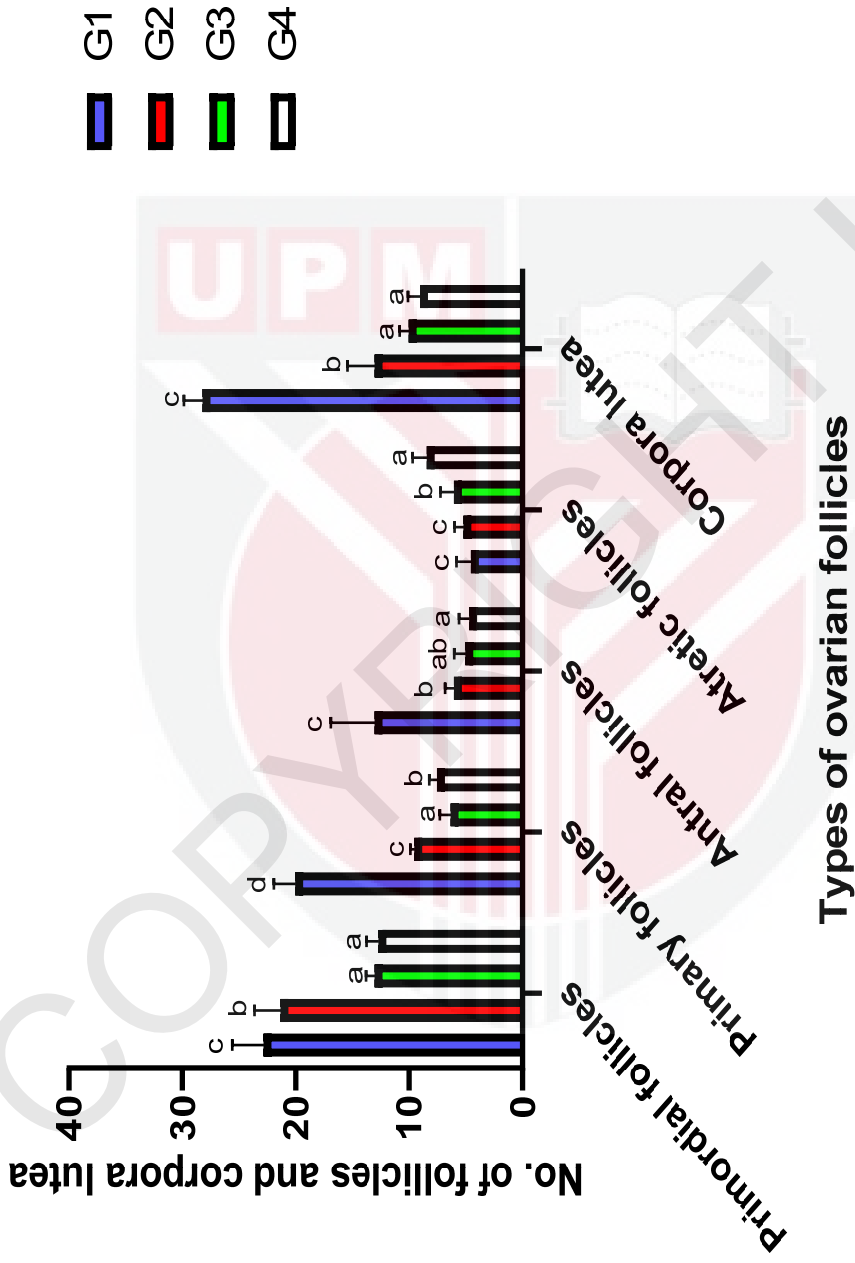


Figure 4.8: IMC's impact on follicular growth in female rats following exposure

The data are expressed as Mean $\pm$ SEM. The error bars that show different letters a, b, c denotes significant difference at $p < 0.05$. *G1=Control (Blue), G2= 4.33mg/kg bwt (Red), G3= 4.66mg/kg bwt (Green), G4= 5.00mg/kg bwt (White).

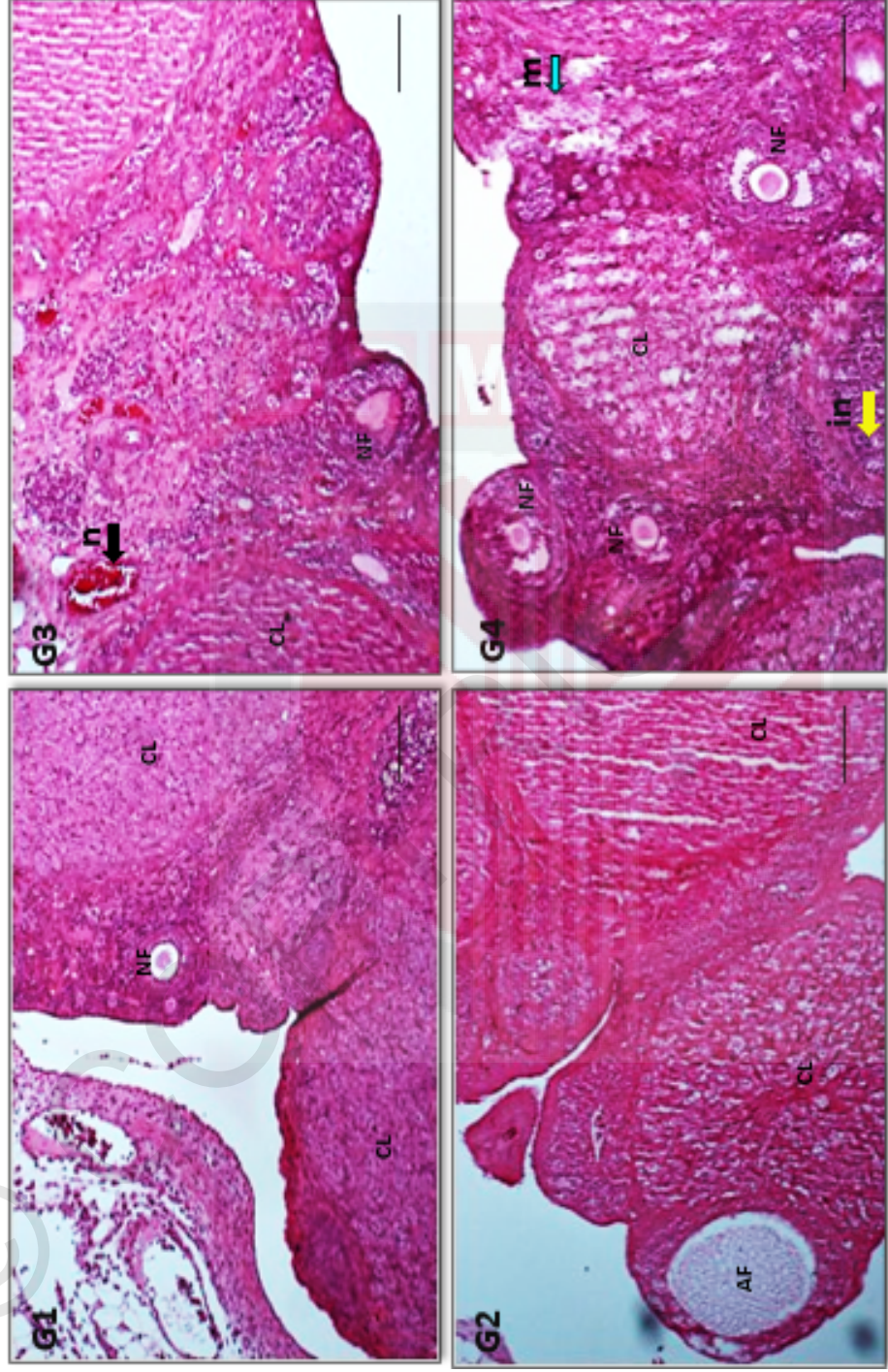


Figure 4.9: Photomicrographs of the ovaries of rats exposed to different doses of IMC toxicity

Here, a micrograph of the ovaries of the control group (G1) is compared to the treated groups (G2, G3, and G4) showing areas of inflammatory alterations (in) with a yellow arrow, necrosis (n) with black arrow and ovarian atrophy (m), with a blue arrow. H&E stain. 400x magnification.

Note: Antral follicle = NF; atretic follicles = AF; corpus luteum = CL.

4.3.4 Uterine histomorphological analysis

During this investigation, no gross abnormal lesions were discovered within the uterus. Histological analysis of uteri of three considered treatment groups however demonstrated the dose-dependent pathological alterations caused by IMC. Except for the control group, the remaining groups demonstrated IMC-induced inflammatory alterations defined by mild endometrial oedema and detachment of endometrial stromal cells from the uterine mucosa. Additionally, as illustrated in Figure 4.10, a decreased number of endometrial glandular epithelia, atrophy of the smooth muscle cell layers, and micro vacuolization of the endometrial layer were found in the 4.66 mg/kg bwt and 5.00 mg/kg bwt of the IMC group. Mean scores of histopathological lesions in the uterus show that there is a significant infiltration of inflammatory cells in 4.33 mg/kg bwt, 4.66 mg/kg bwt groups and congestion in 4.33 mg/kg bwt, 4.66 mg/kg bwt, and 5.00 mg/kg bwt of IMC groups. Also, there is significant necrosis present in luminal epithelium and glandular epithelium of ovarian tissues in all groups (Table 4.2). Furthermore, Figure 4.11 showed the histopathological analysis of the uterine tissue, where vasoconstriction and fibrosis in uterine tissues of 4.66 mg/kg bwt and 5.00 mg/kg bwt of IMC were noticed when compared to considered control group.

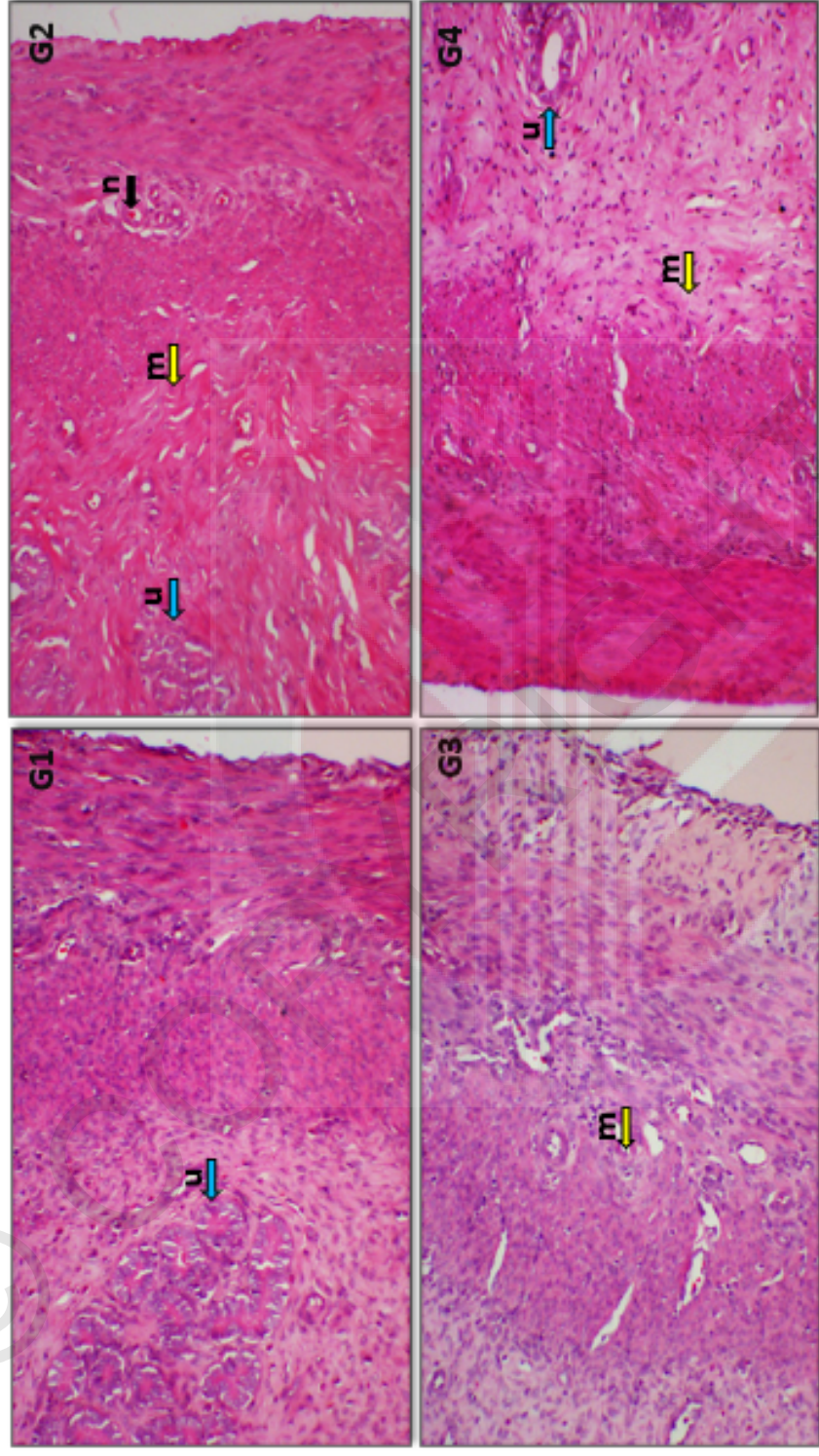


Figure 4.10: Photomicrographs of uterus of rats exposed to different doses of IMC toxicity

Here, the micrograph of the uterus of rat of control group (G1) compared to treated groups (G2, G3, G4) shows the areas, necrosis with a black arrow and smooth muscle atrophy (m) with a yellow arrow, and the uterine gland (u) represented by the blue arrow. H&E stain. 200x magnification.

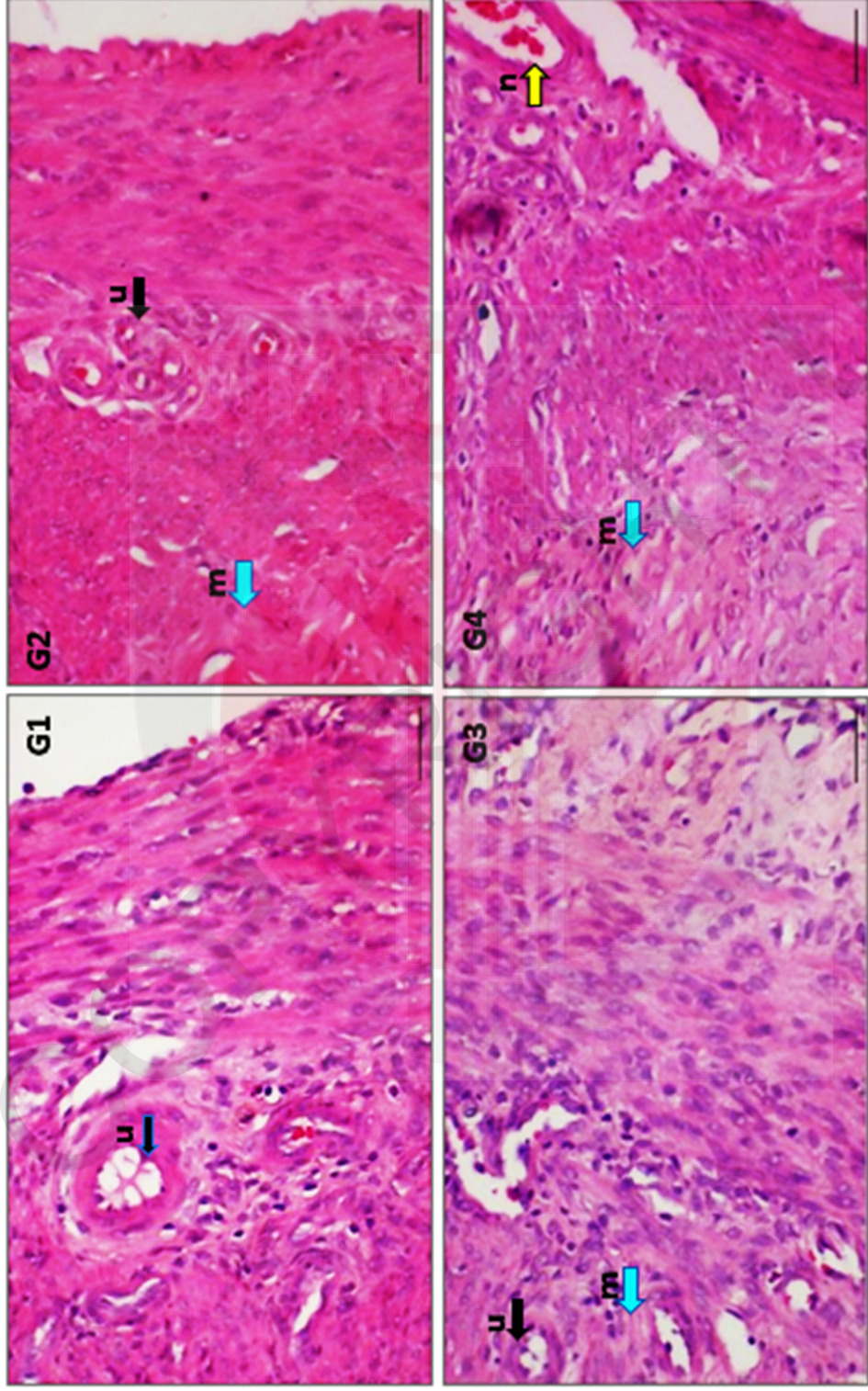


Figure 4.11: Photomicrographs of uterus of rats exposed to different doses of IMC toxicity.

Here, the micrograph of the uterus of rat of control group (G1) compared to treated groups (G2, G3, G4) shows the areas of necrosis (n) with a yellow arrow, smooth muscle atrophy (m) with a blue arrow, and the uterine gland (u) represented by the black arrow. H&E stain. 400x magnification.

Table 4.2: Mean ± SEM scores of histopathological lesions in uterine and ovarian tissues of rats exposed to IMC toxicity

Parameters	Uterus				Ovary			
	Groups							
	G1	G2	G3	G4	G1	G2	G3	G4
Vacuolation	0±0 ^a	1.67±0.56 ^b	1.83±0.2 ^b	2.33±0.58 ^c				
Necrosis	0±0 ^a	1.31±0.58 ^a	2.33±1.15 ^c	2.33±1.15 ^c	0±0 ^a	1.33±0.58 ^a	1.67±0.58 ^b	2.33±0.57 ^c
Infiltration of inflammatory cells	0±0 ^a	1.33±0.58 ^b	2.33±0.57 ^c	2±1 ^c	0±0 ^a	1.83±0.2 ^b	2±1 ^c	2.67±0.58 ^d
Congestion	0±0 ^a	1.33±0.58 ^b	1.67±0.57 ^b	2.67±0.3 ^c	0±0 ^a	1.67±0.58 ^b	2±1 ^c	2.83±0.29 ^d

Means with different superscripts within each row differ significantly (P<0.05). Data are expressed as Mean ± SEM.

4.3.5 Histomorphological analysis of kidney

SD rats were given dosage values of 4.66 and 5.00 mg/kg (groups G3 and G4, respectively) developed a degenerative Bowman's capsule, exhibiting necrosis and inflammatory cell formation, as illustrated in Figure 4.12. When compared to the treated groups, light microscopy analysis of kidney samples from G1 revealed a normal histological structure of renal parenchyma. Mean scores of histopathological lesions in the kidney show that there is a significant necrosis present in the renal tissues in the 4.66 mg/kg bwt of the IMC group (Table 4.3).

4.3.6 Liver morphological analysis

The liver morphology study for the group 5.00 mg/kg bwt of the IMC group revealed microvacuolated hepatocytes, necrosis, and inflammatory cell infiltration. Whereas, as shown in Figure 4.13, vasoconstriction of hepatocytes was identified in the treatment groups at 4.33 mg/kg bwt and 4.66 mg/kg bwt of the IMC. With light microscopy examination of liver tissues from the control group, a typical structural organization, abundant binucleated hepatocytes, absence of inflammatory cell infiltration, and normal central veins were found (G1). Mean scores of histopathological lesions in the liver showed that there was a significant activation of the Kupffer cells in the 4.33 mg/kg bwt, 4.66 mg/kg bwt and 5.00 mg/kg bwt of the IMC groups (Table 4.3 and 4.4).

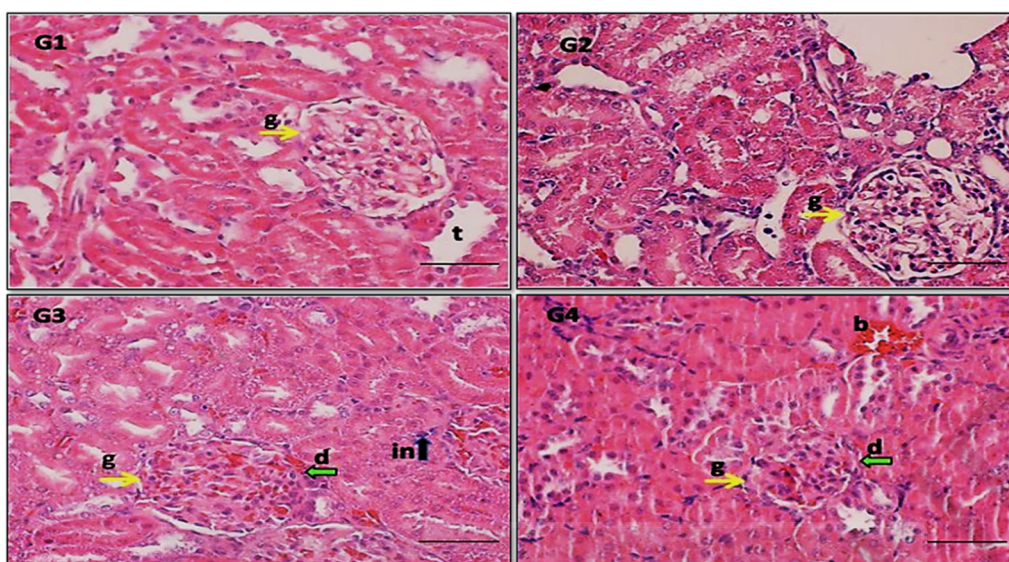


Figure 4.12: Micrographs of the kidney rats exposed to different doses of IMC toxicity

Here, micrograph of the kidney of control group (G1) shows the normal histological structure of renal parenchyma, when compared against the treatment groups (G2, G3, G4) showing congestion of inter tubular blood vessels (b), degenerative bowman's capsule (d), with a green arrow, inflammatory cells (in), and glomerulus (g) represented with a yellow arrow. H&E stain. 400x magnification.

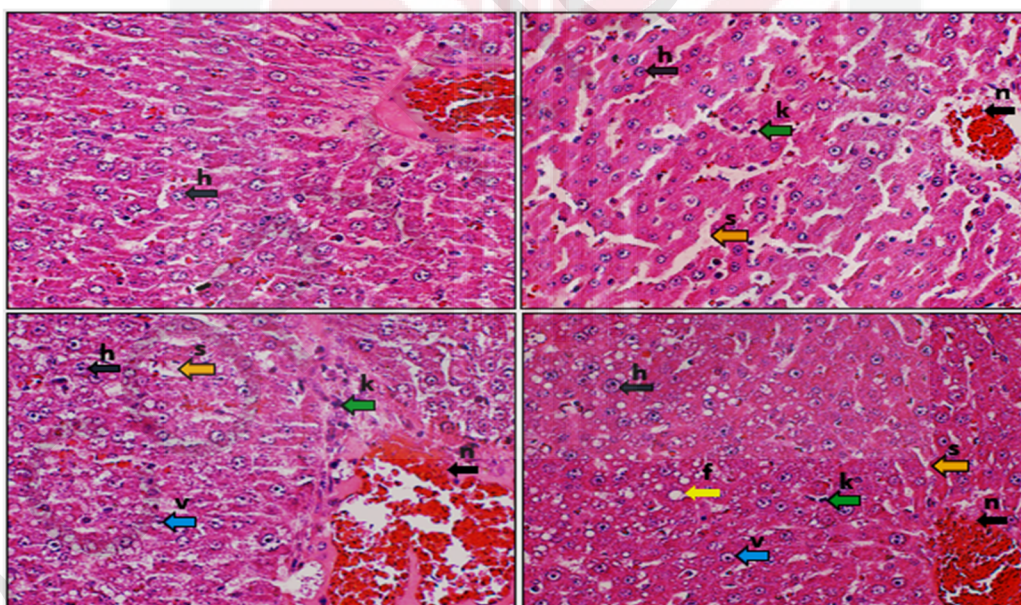


Figure 4.13: Micrographs of the liver of rats exposed to different doses of IMC toxicity

Here, micrograph of the liver of control group (G1) compared to treatment groups (G2, G3, G4). Hepatocytes are represented with a yellow arrow fat lobules (f), vacuolation of hepatocytes (v) with a blue arrow, sinusoidal dilatation (s) with orange arrow, hepatocytes (h) with grey arrow, kupffer cells (k) with green arrow and the necrosis (n) are represented with a black arrow. H&E stain. 400x magnification.

Table 4.3: Mean $\pm$ SEM scores of histopathological lesions found in the kidneys of rats supplemented with EBN and exposed to IMC toxicity

Parameters	Kidney			
	G1	G2	G3	G4
*Vacuolation	0 $\pm$ 0 ^a	1 $\pm$ 0 ^a	1.3 $\pm$ 1.5 ^b	2 $\pm$ 1 ^c
*Necrosis	0 $\pm$ 0 ^a	2 $\pm$ 0 ^b	2.3 $\pm$ 0.5 ^c	2.3 $\pm$ 1.2 ^c
*Infiltration of inflammatory cells	0 $\pm$ 0 ^a	2 $\pm$ 0 ^b	2.33 $\pm$ 0.5 ^c	2 $\pm$ 1 ^c
*Congestion	0 $\pm$ 0 ^a	1.3 $\pm$ 0.5 ^a	1.67 $\pm$ 0.5 ^b	3 $\pm$ 0 ^c
*Atrophy of the glomerulus	0 $\pm$ 0 ^a	1 $\pm$ 0 ^a	1.6 $\pm$ 0.5 ^b	2.6 $\pm$ 0.5 ^c

Within the same row, mean scores with changed superscripts are considerably different from each other at $p < 0.05$. Data are expressed as Mean $\pm$ SEM.

Table 4.4: Mean $\pm$ SEM scores of histopathological lesions found in liver of rats supplemented with EBN and exposed to IMC toxicity

Parameters	Liver			
	G1	G2	G3	G4
*Vacuolation	0 $\pm$ 0 ^a	1.3 $\pm$ 0.5 ^b	1.3 $\pm$ 1.5 ^b	2.1 $\pm$ 0.2 ^c
*Necrosis	0 $\pm$ 0 ^a	1.6 $\pm$ 0.5 ^b	2.3 $\pm$ 1.1 ^b	2.5 $\pm$ 0.5 ^c
*Infiltration of inflammatory cells	0 $\pm$ 0 ^a	2.3 $\pm$ 1.5 ^b	2 $\pm$ 0 ^b	3 $\pm$ 0 ^c
*Congestion	0 $\pm$ 0 ^a	1.3 $\pm$ 1.5 ^b	1.6 $\pm$ 0.5 ^b	2.6 $\pm$ 0.5 ^c
*Activation of Kupffer cells	0 $\pm$ 0 ^a	1.3 $\pm$ 0.5 ^b	1.6 $\pm$ 0.5 ^b	2.6 $\pm$ 0.5 ^c

Within the same row, mean scores with changed superscripts are considerably different from each other at $p < 0.05$. Data are expressed as Mean $\pm$ SEM.

4.4 Discussion

The majority of NSAIDs have been found to have a detrimental effect on PG production by inhibiting COX1 and COX2 enzymatic activity (Dannhardt & Kiefer, 2001). Lethargy, abdominal oedema, and decreased appetite are observed as indicators of IMC-induced toxicity (Kaplan et al., 2012). In this investigation, SD rats were given 4.66 and 5.00 mg/kg bwt of IMC on Pd3 and Pd4, respectively, demonstrating maternal toxicity. This data is consistent with discoveries of the research presented by (Damasceno et al., 2008), which demonstrated a decrease in feed intake and bwt from Pd6 to Pd11 in rats given an IMC dose of 8.50 mg/kg bwt. IMC, a COX-inhibiting

NSAID, has also been shown to be useful in reducing EIR (Ikeda et al., 2019). Participation of PGs in the early stages of implantation, which is similar to any other inflammatory response (Ricciotti et al., 2011). Additionally, the toxic damage to the uterine tissue generated by IMC may share to loss of implantations in treated groups by impairing the endometrial cells' ability to make PG (Damasceno et al., 2008). IMC's action has been attributed to its capacity to inhibit PG synthesis within the uterus (Bergström, 2014). Furthermore, inhibiting COX-2 results in a restrictive response on blastocyst implantation, whereas inhibiting both COX-1 and COX-2 isoforms simultaneously due to IMC supplements results in greater detrimental effects on implantation (Sookvanichsilp & Pulbutr, 2002). Numerous miscarriages, including defects in ovulation, implantation, and fertilization, have been directly linked to female COX-2 deficiency (Song et al., 2000). Another study revealed smooth muscle atrophy and vasoconstriction within the uterus following IMC treatment, resulting in decreased smooth muscle contraction and contraction within the uterus (Mohan et al., 2006). Thus, it can be established that IMC in a sufficient dosage is capable of establishing EID.

However, the majority of research that has been published so far has focused on negative consequences of IMC on the Wister rat mammalian reproductive health. There is, nevertheless, less research on the effectiveness of IMC treatment in establishing EID in SD rats. When IMC was supplied at a dosage of 5 mg/kg on Pd3 and Pd4 and 10 mg/kg/day from Pd1 to Pd7, a decreased number of normal implantation sites was detected in an experiment conducted by (Sookvanichsilp & Pulbutr, 2002). Even when IMC was provided subcutaneously at a dosage value of 1 mg/kg twice on the Pd5, no considerable changes in number of implantation's were

found (Kennedy, 1977). In this case, the use of IMC on Pd5 instead of Pd3 maybe held accountable for the decreased effectiveness of IMC.

The number of EIR was reduced in studies using IMC dosages of 3 and 5 mg/kg bwt delivered twice daily from Pd3 to Pd5 (Phillips & Poyser, 1981; Poyser & Acids, 1999). Toxic gastrointestinal haemorrhages, even fatalities, were reported at a dose of 8.40 mg/kg/day and 10 mg/kg/day (Shafiq et al., 2004; Damasceno et al., 2008; Salleh, 2014). A dose of 10 mg/kg/day, both the pre- and post-implantation losses rose significantly (Shafiq et al., 2004). Wister rats' reproductive health was unaffected at lower dosages of 0.32, 1, and 1.68 mg/kg bwt twice daily from Pd3 to Pd5 (Kennedy 1977; Damasceno et al., 2008). EIR was significantly reduced in all groups of rats given IMC treatment in the current investigation. The dose of 4.33 mg/kg resulted in a small number of EIR, although the liver and kidney were not adversely affected to a significant degree. As a side effect of treating IMC, vasoconstriction and vascularization were observed in the uterine tissues. According to the findings of this study, the IMC's restriction of PGs is the cause of the vasoconstriction in uterine tissues. It's still consistent with the findings of (Ekamen et al., 2008). Aside from ischaemia, smooth muscle atrophy can be a direct result of a loss of blood flow to the muscle (Bishop-Bailey & Warner, 2003). High doses of an NSAID have been shown to alter the walls of uterine horns and lower the capacity of the lumen and the layers of the lamina propria and the epithelium (Güven et al., 2013). Histopathologically, vasoconstriction, dilatation, and intertubular blood vessels congestion along with a degenerative Bowman's capsule were discovered histopathologically in the present study. In contrast to these pathological findings, the treated SD rats in G2 had no changes to their kidneys other than microvacuolation in their epithelial linings. Progesterone levels and the number of corpora luteum increased in G1 when compared to treated groups, indicating that the number of corpora luteum also increased.

According to prior investigations, the vasoconstriction of portions of the ovaries was an indication of blocked blood arteries. Rats treated with IMC at a dose 3.5 and 4mg/kg showed vasoconstriction in the ovaries (Ekanem, Garba, & Jankada, 2008). Microvacuolization of hepatocytes was confirmed in histological investigation at doses of 5.00 mg/kg and 8.40 mg/kg of IMC. The findings of this investigation were completely in line with those found in (Salleh, 2014; Sookvanichsilp & Pulbutr, 2002). As a result, by analysing the EIR and histomorphological changes in the ovaries, the uterus, kidney, and liver of SD rats in the current study, it can be concluded that IMC has a negative impact on the reproductive health of SD rats at a dose of 4.33 mg/kg bwt or greater.

4.5 Conclusion

The IMC therapy exhibited interference with implantation and embryo development in rats. A dosage of 4.33 mg/kg bwt of IMC was found to be sufficient to induce EID. In comparison to the control group, the IMC groups exhibited vasoconstriction and fibrosis in uterine tissues, with dosages of 4.33, 4.66, and 5.00 mg/kg bwt. Consequently, extensive vasoconstriction and smooth muscle atrophy were observed in the uterus. Notably, the histopathological analysis of kidney tissue in the 4.66 mg/kg bwt IMC group revealed significant necrosis. Moreover, the histopathological examination of liver tissue indicated a notable activation of Kupffer cells in the IMC groups with dosages of 4.33, 4.66, and 5.00 mg/kg bwt. Therefore, it can be concluded that a dosage of 4.33 mg/kg bwt of IMC is sufficient to induce EID.

CHAPTER 5

PROPHYLACTIC EFFECT OF EDIBLE BIRD'S NEST ON INDOMETHACIN INDUCED REPRODUCTIVE TOXICITY

5.1 Introduction

In the modern world, when many factors could endanger fertility and reproductive health, reproductive toxicity is a major worry. NSAIDs like IMC, which are frequently used for their analgesic and anti-inflammatory characteristics, are one such reason. These medications have been linked to negative effects on reproductive function, including implantation failure, impaired embryo development, and tissue damage, despite being helpful in treating pain and inflammation. There is consequently a rising need to investigate alternate strategies to lessen the reproductive damage linked to NSAID use.

The natural food known as edible bird's nest, which is made from the saliva of swiftlets, has drawn interest due to its traditional use in fostering fertility and reproductive health as well as its possible health advantages. Numerous bioactive substances, including minerals, amino acids, and glycoproteins, are known to be present in it; these substances may help explain its alleged therapeutic properties. Even while anecdotal data shows that edible bird's nest may have anti-reproductive toxicity characteristics, empirical study in this field is still in its infancy.

People are exposed to a wide range of chemical contaminants on a daily basis. Due to their permeable blood-brain barrier and high gastrointestinal absorption, children are

particularly susceptible to IMC exposure. Reactive oxygen species, nitrogen oxides, and lipid peroxidation markers like malondialdehyde have all been linked to oxidative stress and antioxidant imbalance, and all of these conditions are made worse by lipid peroxidation inhibitors like glutathione. As a result, research into the genetic and pharmacological processes of IMC-induced reproductive impairment is critical (Rzymiski et al., 2015). There is a strong correlation between IMC exposure and infertility in dairy animals, as well as malnutrition and ovulatory or hormonal abnormalities (Mason et al., 2022). Less research has been done regarding effects of the environmental contaminants on the female reproductive health and how this affects breeding. Long-term exposure to IMC, on the other hand, may impede ovulation, follicular growth, and menstruation in other animals, all of which are regulated by uterine gland secretions (Gulati et al., 2022). Ovarian implantation and pregnancy in mice may be less common, which may delay pubertal rats' vaginal opening (Jain & Singh, 2021). Physiotherapists have recently been introduced to use of the medicinal foods with an antioxidant activity to shield them from IMC toxicity (Singh et al., 2014). Edible bird's nest (EBN), which offers medical benefits (anti-aging, anti-cancer, immunity-boosting, and antioxidant) as well as nutritional value (water-soluble protein, carbohydrate, iron, and fibre) for defence against harmful toxins, is gaining more and more attention from researchers. EBN has been linked to a number of therapeutic outcomes. One of its most frequently expressed opinions is that it improves sexual ability or reproduction, which requires scientific data to be supported. Therefore, the uterine glandular and luminal epithelium (GE and LE), expression of epidermal growth factor (EGF), EGF receptor (EGFR), vascular endothelial growth factor (VEGF), proliferating cell nuclear antigen (PCNA), and steroid receptors on uterine and ovarian tissue, and oxidative stress biomarker (O) should be the primary foci of a study on the protective effect of EBN treatment on IMC administration.

5.2 Methods

5.2.1 EBN and IMC preparation

Preparation of the EBN and IMC was carried out in the same manner as stated in Chapter 3.

5.2.2 Experimental design

Six female Sprague-Dawley rats (12 weeks old) were housed in five to six cages. In this study, rats were randomised into five groups (with six animals in each) after seven days of acclimation, and then were given IMC for eight weeks while also receiving in vivo edible bird's nest supplements as shown in Table 5.1. The amount of EBN was calculated by Ismail et al. (2014). Histological analyses of ovarian and uterine tissue were undertaken to check for the expression of EGF, EGFR, VEGF, PCNA, steroid hormone receptors, OSB, and AO, as detailed in Chapter 3.

Table 5.1: Group allotment of the animals and treatment regime

Group	Assignment (6 rats/group)	Type of treatment (dose)/8 weeks
Control	G1 (Control)	Normal diet + Normal saline
Treated Groups	G2 (Positive control)	IMC only (4.33mg/kg)
	G3 (Low dose)	IMC + EBN (4.33mg/kg + 60mg/Kg body wt.)
	G4 (Medium dose)	IMC + EBN (4.33mg/kg + 90mg/Kg body wt.)
	G5 (High dose)	IMC + EBN (4.33mg/kg +120mg/Kg body wt.)

ND=Normal diet, IMC=Indomethacin, EBN=edible bird's nest, bwt=Body weight.

5.2.3 Oestrous synchronization and the determination of oestrous cycle phases

Oestrous synchronisation and the determination of the oestrous cycle phases followed as described in Chapter 3.

5.2.4 Macro and microscopic inspections of the uterine and ovarian tissues

Macro and microscopic inspections of reproductive tract were accomplished as described in chapter 3.

5.2.5 SEM inspections of the uterine and ovarian tissues

SEM inspections of the uterine and the ovarian tissues were carried out in the same method outlined in Chapter 3.

5.2.6 The Analyses of expressions of the EGF, EGFR, PCNA, VEGF, E2R, and P4R in the uterine and ovarian tissues by IHC

Using the methods outlined in Chapter 3, we analysed the expression levels of EGF, EGFR, PCNA, VEGF, E2R, and P4R in uterine and ovarian tissues using IHC.

5.2.7 Hormone assays and Blood collection

Hormone assays (E₂= Estradiol hormone, P₄= Progesterone hormone) and Blood plasma collection by using ELISA approach were done as described in chapter 3.

5.2.8 Assessment of plasma concentration of SOD and TBARS levels

Analyses of the plasma concentration of SOD and TBARS were completed as detailed in Chapter 3.

5.2.9 Statistical analysis

The standard error of the mean (SEM) around the mean data was analysed in Graph Pad Prism 6.0. Graph Pad Software is situated in sunny San Diego. The assumption of normality was tested with Shapiro-Wilk. The number of ovarian and uterine glands, hormone levels, oxidative stress biomarkers (OSB), and AO, as well as ratios and lengths of the ovarian and uterine bodies, were evaluated using an ANOVA with the Tukey multiple comparison post-hoc test. Meanwhile, the body weights were compared using a two-way ANOVA followed by Bonferroni's multiple comparison testing. A non-parametric statistical test (Kruskal-Wallis) followed by post hoc Mann Whitney U test for multiple comparison, was used to compare the expression of steroid receptors, growth factors, PCNA, and the thickness of the LE, GE, and endothelium. The significance level was less than 0.05.

5.3 Results

5.3.1 The Effect of EBN on the body weight gain (BWG)

Although having typical (4-5 day) oestrous cycles, rats exposed to IMC all underwent significant changes in bwt during the course of the trial. When compared to the negative control group at week 8, the bwt was seen to be considerably lower in the positive control group (G2) and significantly higher ($p < 0.05$) in the higher dose (G5) of the edible bird's nest group (Figure 5.1).

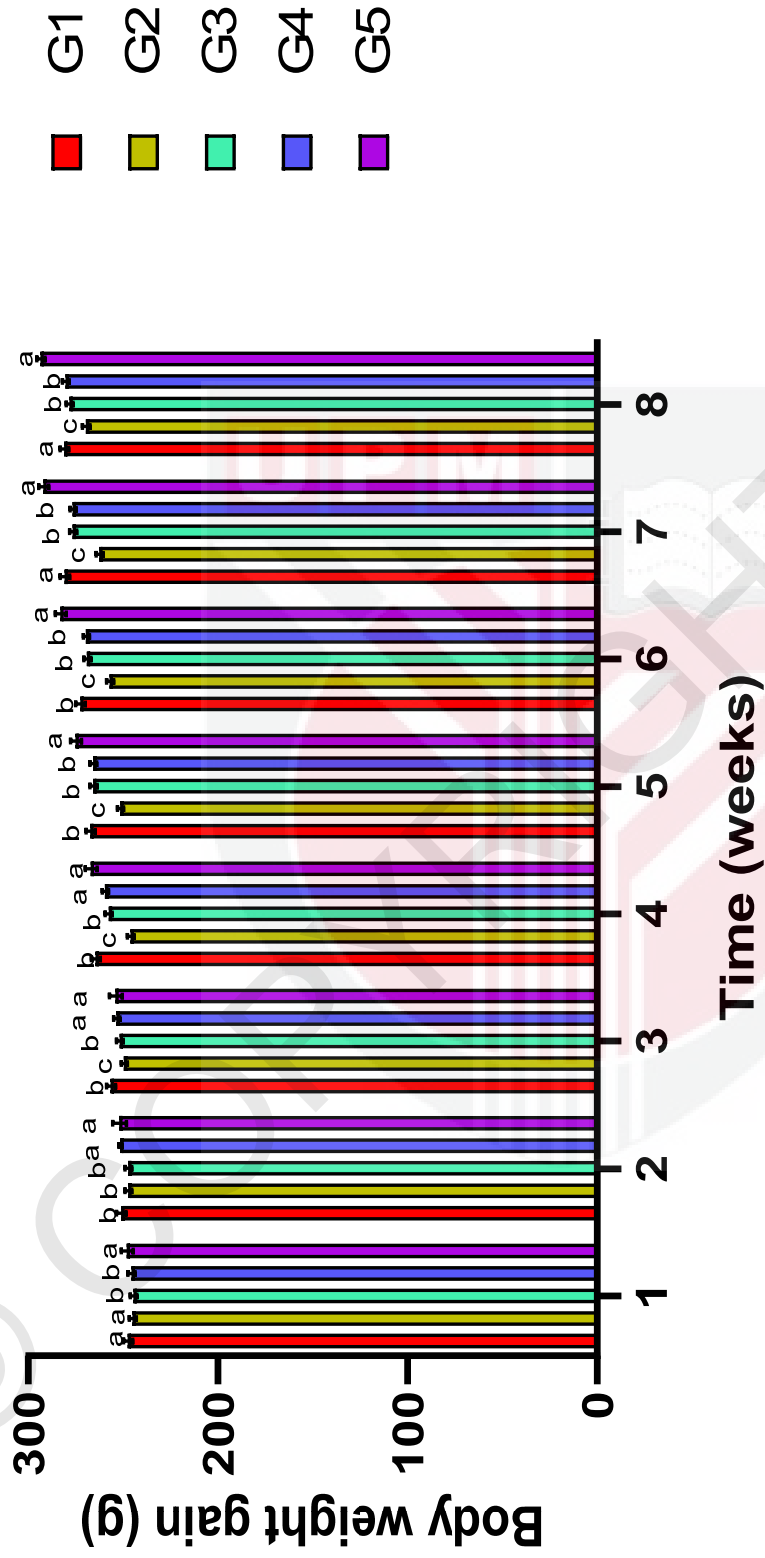


Figure 5.1: Body weight gain (g) of EBN treated groups compared to control and IMC groups.

Data are expressed as Mean±SEM. Error bars show different letters a, b, c denotes significant difference at $p < 0.05$. *G1=Control, G2= (4.33mg/kg bwt), G3= (4.33+60mg/kg bwt), G4= (4.33+90mg/kg bwt), G5= (4.33+120mg/kg bwt).

5.3.2 The Effect of EBN on the uterine and ovarian: bwt ratio (UBWR) & (OBWR)

Furthermore, the UBWR and OBWR were found significantly lesser in positive control (G2) as well as significantly greater ($p<0.05$) with high dose (G5) when correlated to treatment, positive and then negative control groups (as displayed in the Figure-5.2A and Figure-5.2B).

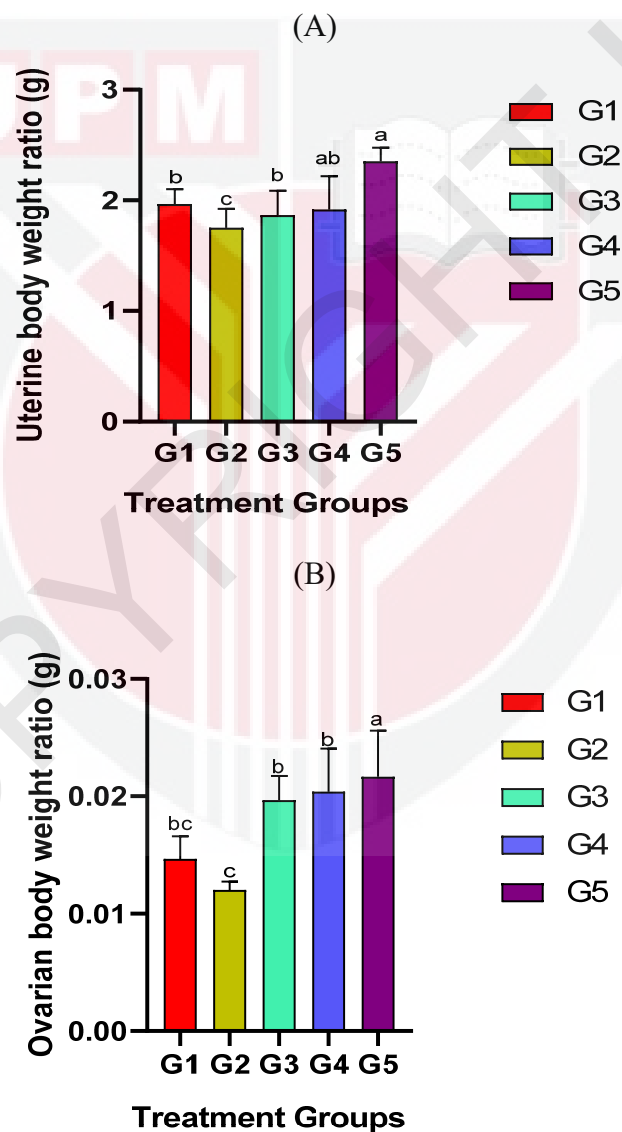


Figure 5.2: UBWR (A) and OBWR (B) of EBN treated groups when compared to control and IMC groups

Data are expressed as Mean $\pm$ SEM. Error bars show different letters a, b, c denotes significant difference at $p<0.05$. *G1=Control, G2= (4.33mg/kg bwt), G3= (4.33+60mg/kg bwt), G4= (4.33+90mg/kg bwt), G5= (4.33+120mg/kg bwt).

EBN increased the uterus length and weight dose-dependently. Luminal epithelium thickness decreased in IMC treated group (G2) in comparison to 120mg/kg bwt (G5) of the EBN group. IMC raised LE thicknesses in a dose-dependent manner. The higher LE thicknesses in all treated groups were substantially ($p<0.05$) higher than lower in treated groups 90 mg/kg bwt (G4) and 120mg/kg bwt (G5) of the EBN (Figure-5.3).

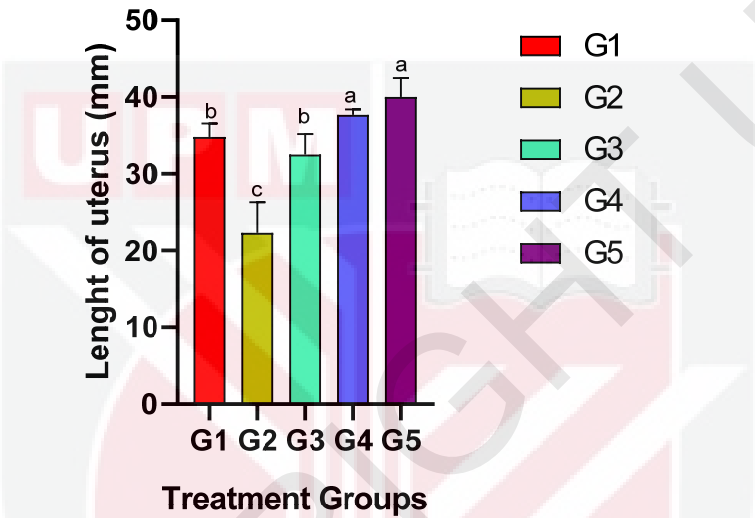


Figure 5.3: Uterine length (UL) of EBN treated groups when compared to control and IMC group

Data are expressed as Mean±SEM. Error bars that show different letters a, b, c denotes significant difference at $p<0.05$. *G1=Control, G2= (4.33mg/kg bwt), G3= (4.33+60mg/kg bwt), G4= (4.33+90mg/kg bwt), G5= (4.33+120mg/kg bwt).

5.3.3 Embryo implantation rate and Fertility index

On day 8 of pregnancy, the fertility index and the number of embryo implantation rates (EIR) were compared between the control group and the rats given EBN supplements. Comparison of EBN members' reproductive rates and EIR counts positive control (G2) and 60mg/kg bwt (G3) were not statistically significant, However, compared to the other treatment groups, there was a statistically significant difference between the control group (G1) and the highest EBN-treated group (Figure-5.4). Figure-5.4(A) and

(B) shows that when comparing the implantation rates of embryos from different groups, the control group (G1) and the 120mg/kg bwt EBN group (G5) both performed the best.

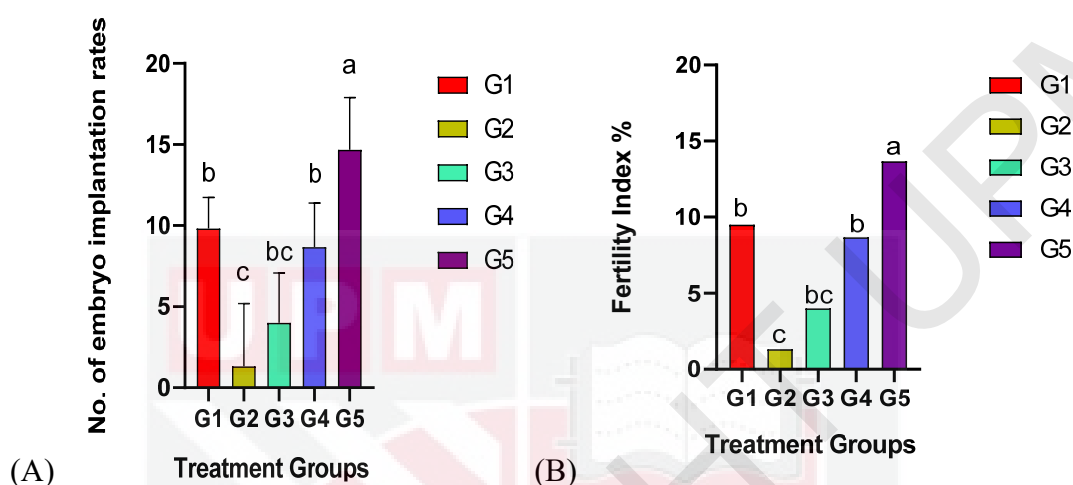


Figure 5.4: Effects of IMC and EBN on embryo implantation rates (A) and fertility index %; (B)

Data are expressed as Mean±SEM. Error bars that show different letters a, b, c denotes significant difference at $p < 0.05$. *G1=Control, G2= (4.33mg/kg bwt), G3= (4.33+60mg/kg bwt), G4= (4.33+90mg/kg bwt), G5= (4.33+120mg/kg bwt).

5.3.4 Histopathological examination of uterine and ovarian tissues using light microscope

The uterine and ovarian tissues were examined, but no obvious gross pathological abnormalities were discovered. However, histopathological examination of the uteri of the various groups (apart from the positive control and the groups receiving 60 mg/kg bwt of edible bird's nest). Lower and medium EBN treatment groups showed inflammatory changes, while IMC-induced groups showed uterine lumen thickening, endometrial atrophy, vacuolar degeneration. The uterine structure of the edible bird's nest groups, however, was identical to the control group at the maximum dose of 120 mg/kg bwt (G5). When compared to the positive control (G2) and the other EBN treated groups, the highest dose (G5) of the edible bird's nest group samples shown

exceptional development and a bigger quantity of endometrial structures (Figure-5.5). The positive control (G2), 60 mg/kg bwt (G3), and 90 mg/kg bwt (G4) of the edible bird's nest groups, however, demonstrated ovarian tissue atrophy in comparison to the 120 mg/kg bwt (G5) of the edible bird's nest group upon histological examination of the ovaries (Figure-5.6). Also, Figures 5.2 and 5.3 display the mean score differences of histological lesions in the uterus and ovary among the groups are shown in the Tables 5.2 and 5.3.



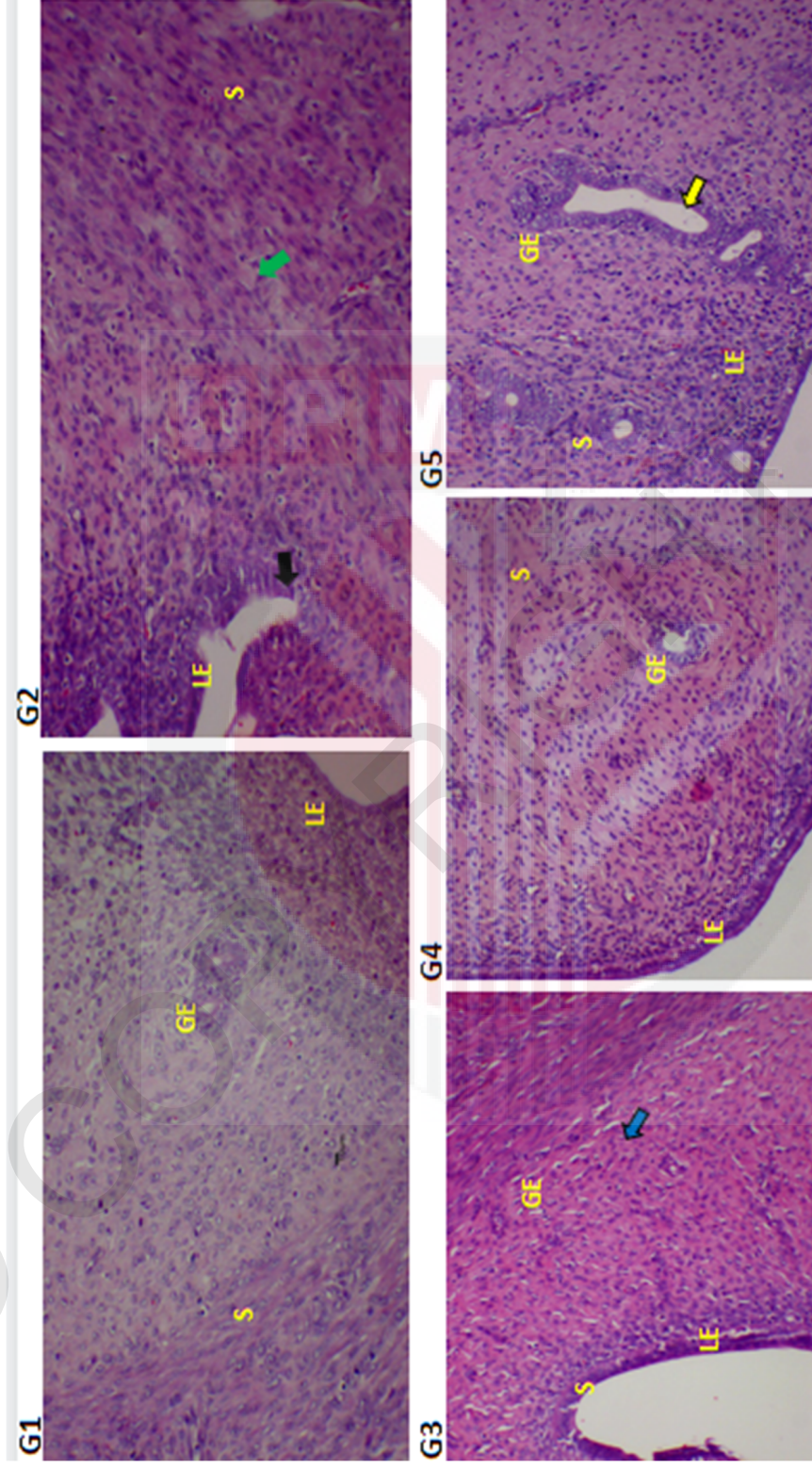


Figure 5.5: Photomicrographs of uterus on the impact of IMC and EBN

*(G1) Control (G2) Positive Control (IMC 4.33 mg/kg bwt); G3 (IMC + EBN 60 mg/kg bwt); G4 (IMC + EBN 90 mg/kg bwt); G5 (IMC + EBN 120 mg/kg bwt). (LE) signify luminal epithelium, (S) stroma and (GE) Glandular Epithelium. Normal uterine glands are indicated by yellow arrows, but vasoconstriction is indicated by (blue arrows) and endometrial atrophy by (green arrows) in G2. Deterioration of LE cells (black arrow) is manifest in G2 compared to treatment groups. H&E stain. Magnification 200x.

Table 5.2: Effect of different levels of edible bird’s nest (EBN) fed to indomethacin (IMC)-exposed rats on histological lesions of the ovary (Mean±SEM)

Parameters	Groups				
	G1	G2	G3	G4	G5
Vacuolation	0±0 ^a	2.3±0.6 ^c	1.3±0.3 ^b	1.1±0.4 ^b	0±0 ^a
*Necrosis of LE and GE	0±0 ^a	2.6±0.3 ^c	1.8±0.1 ^b	0.6±0.1 ^a	0.3±0.1 ^a
*Infiltration of inflammatory cells	0±0 ^a	2.1±1.6 ^c	2.3±0.1 ^c	1.3±0.3 ^b	0.3±0.3 ^a
*Congestion	0±0 ^a	2.8±0.2 ^c	1.8±0.1 ^b	1±0 ^a	0±0 ^a

Means with different superscripts within each row differ significantly (P<0.05)



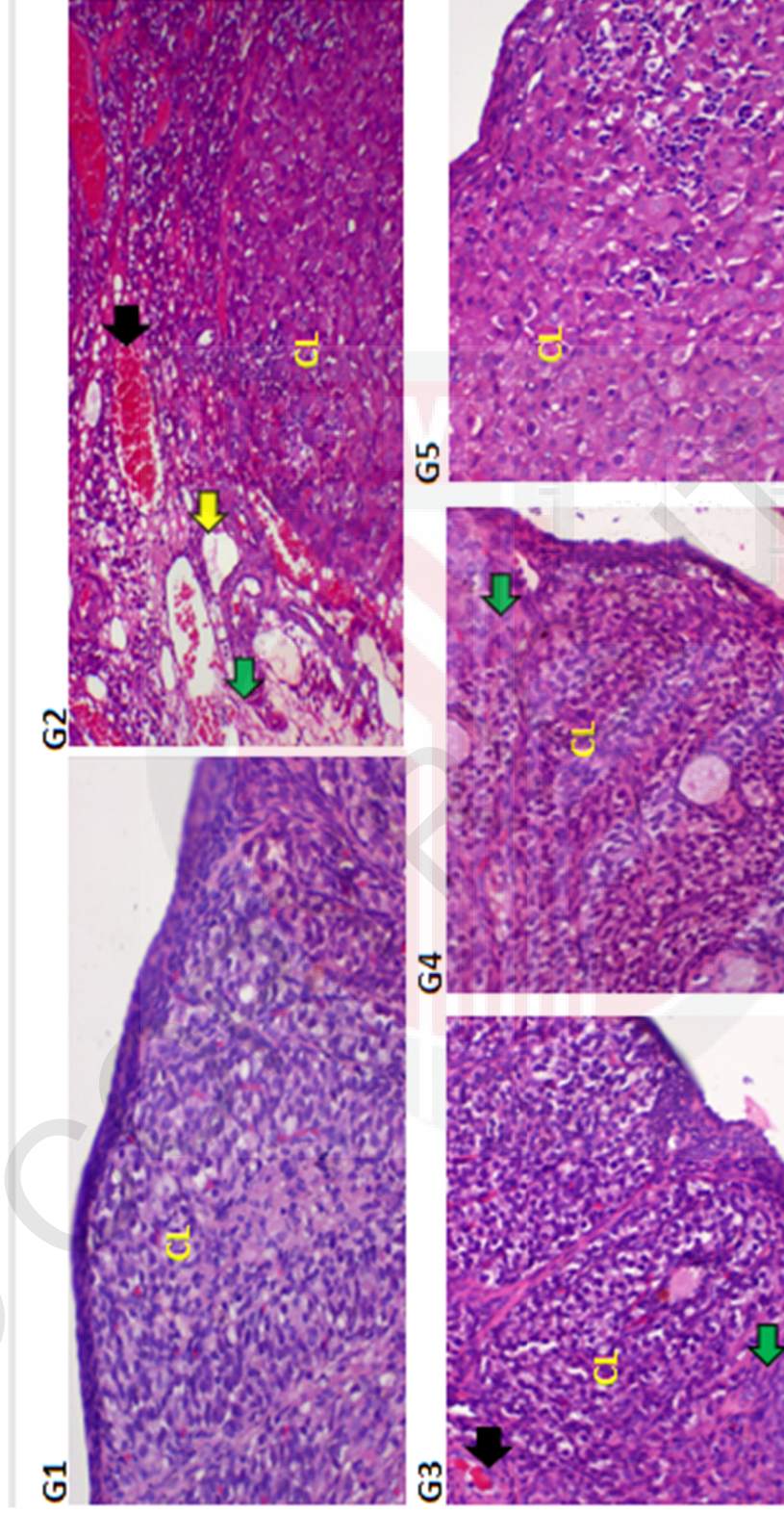


Figure 5.6: Photomicrographs of ovaries on the impact of IMC and EBN

*(G1) Control (G2) Positive Control (IMC 4.33 mg/kg bwt); G3 (IMC + EBN 60 mg/kg bwt); G4 (IMC + EBN 90 mg/kg bwt); G5 (IMC + EBN 120 mg/kg bwt). (CL) signify corpus luteum, (AN) antral follicle. (yellow arrows) indicate fat lobules while (green arrows) indicate congestion, while degeneration of ovarian tissues shown in (black arrow) and ovarian tissue atrophy is evident in G2, G3 and G4 compared to G5 group. H&E stain. Magnification 200x.

Table 5.3: Effect of different levels of edible bird’s nest (EBN) fed to indomethacin (IMC)-exposed rats on histological lesions of the uterus (Mean ± SEM)

Parameters	Groups				
	G1	G2	G3	G4	G5
Vacuolation	0±0 ^a	2.8±0.1 ^c	1.6±0.3 ^b	0.5±0.2 ^a	0±0 ^a
Atrophy	0±0 ^a	2.3±0.3 ^c	2±0 ^c	0.6±0.3 ^a	0±0 ^a
Infiltration of inflammatory cells	0±0 ^a	2.5±0.2 ^c	2±0 ^c	0.8±0.1 ^a	0.5±0 ^a
Congestion	0±0 ^a	3±0 ^c	2.3±0.3 ^c	0±0 ^a	0±0 ^a

Means with different superscripts within each row differ significantly (P<0.05)

5.3.5 Uterine examinations using SEM

The uteri of control and EBN-supplemented rats were found to be different upon microscopic inspection. On Pd8, the uterine epithelium at the locations where the embryos were implanted was smooth with a few wrinkles (Figure-5.7). In contrast to the untreated control and EBN groups, the positive control (G2) group had a lot of folds and irregularly formed pinopods. Ultrastructural SEM examination revealed flat-bordered uterine epithelial cells in the treated groups, with medium-density pinopods in the low and medium EBN dose groups. When compared to positive and other EBN treated groups, high density microvilli and pinopods were found in the groups with the highest EBN levels (Figure-5.7).

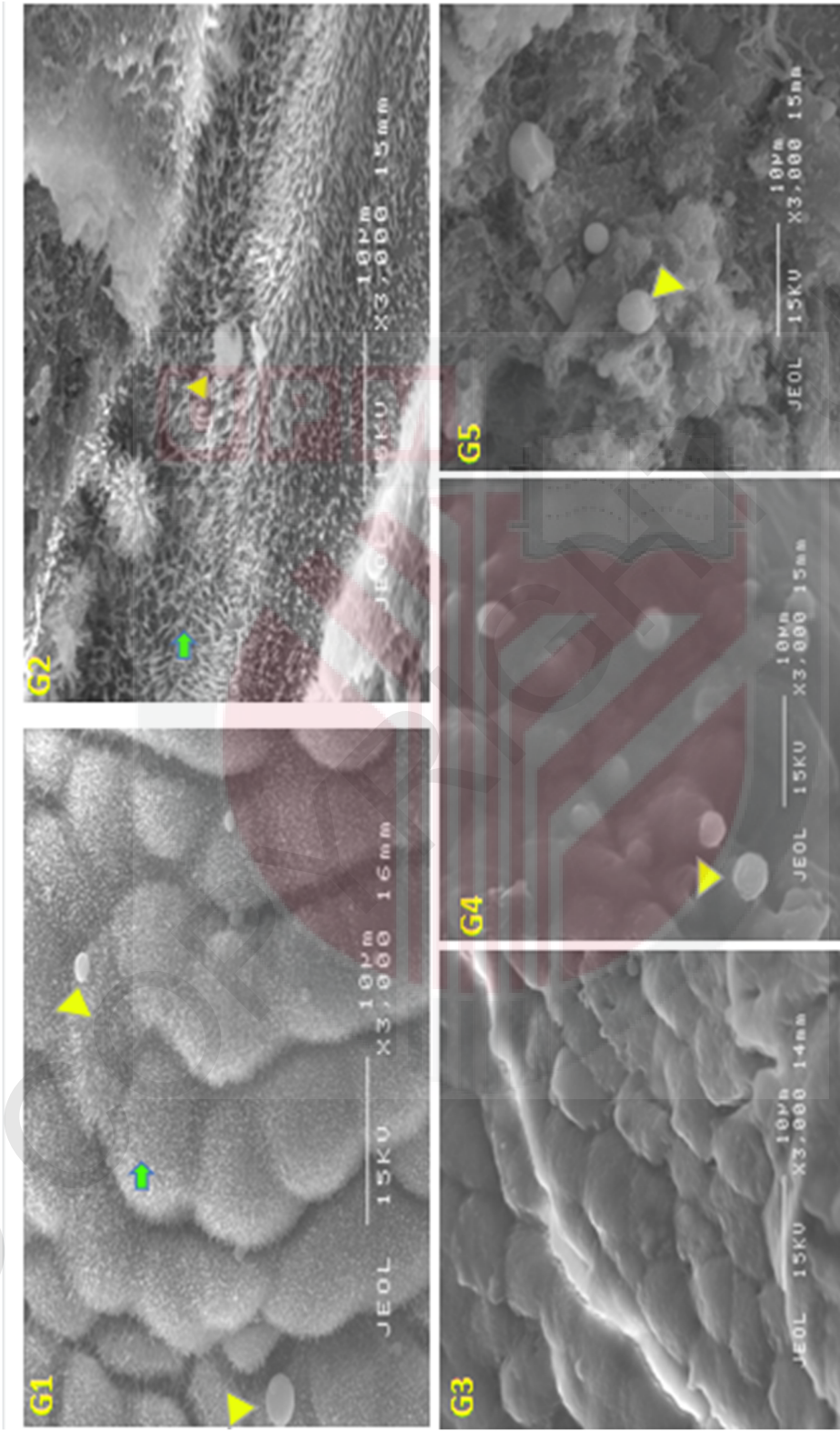


Figure 5.7: Scanning electron micrographs of the uteri of pregnant rats.

Notice, yellow arrow, pinopods, green arrow, microvilli, *G1=Control, G2= (IMC 4.33 mg/kg bwt), G3= (IMC 60 mg/kg bwt) G4= (IMC 4.33 mg/kg, EBN 90 mg/kg bwt) and G5= (IMC 4.33 mg/kg, EBN 120 mg/kg bwt). (Scale bar 10 µm).

5.3.6 Expressions and scoring of EGF, EGFR, PCNA and VEGF in the uterine tissues

Immunohistochemical micrographs (Figures 5.8 - 5.11) provide a descriptive visual representation of the expression of EGF, EGFR, PCNA, and VEGF in uterine sections from all experimental groups. The resulting point spreads are shown in Table 5.4. In the pregnant uterus, increased dosages of edible bird's nest increased stromal cell density and EGFR, VEGF, and PCNA expression. Most measures improved with increasing dose. When compared to the other EBN treatment groups, negative and positive control groups, those who received medium and high doses of edible bird's nest expressed significantly higher at a value of ($p < 0.05$). Weak reactivity with partial staining (nucleus and/or cell membrane) was indicative of expression with EGF in the control group. Nuclear EGF expression was significantly highest ($p < 0.05$) at the highest EBN dose (G5) in S, GE, and LE cells, as shown in (Figure-5.8), when compared to the control and other EBN-treated groups, the EGFR expression scenario was seen with very little to no specific immune reaction found in the positive control (G2); however, significantly higher expressions were seen in the medium (G4) and highest (G5) EBN dose groups, while a low expression was seen in the negative control and lowest EBN dose group, as shown in the slides (Figures. 5.9). Despite the fact that the observed PCNA reaction was significantly ($p < 0.05$) higher in the group illustrated in in the EBN group at a dose 120 mg/kg bwt (G5), when compared to the other EBN dose groups, the negative control group, and the positive control group, the number of S, GE, and LE, as shown in (Figure-5.10). When compared to the negative (G1), positive control (G2), and other EBN-treated groups of VEGFS, VEGF reactions began to be noticed at 120 mg/kg bwt of the EBN supplemented group G5, and were evident throughout the S, GE, and LE (Figure 5.11).

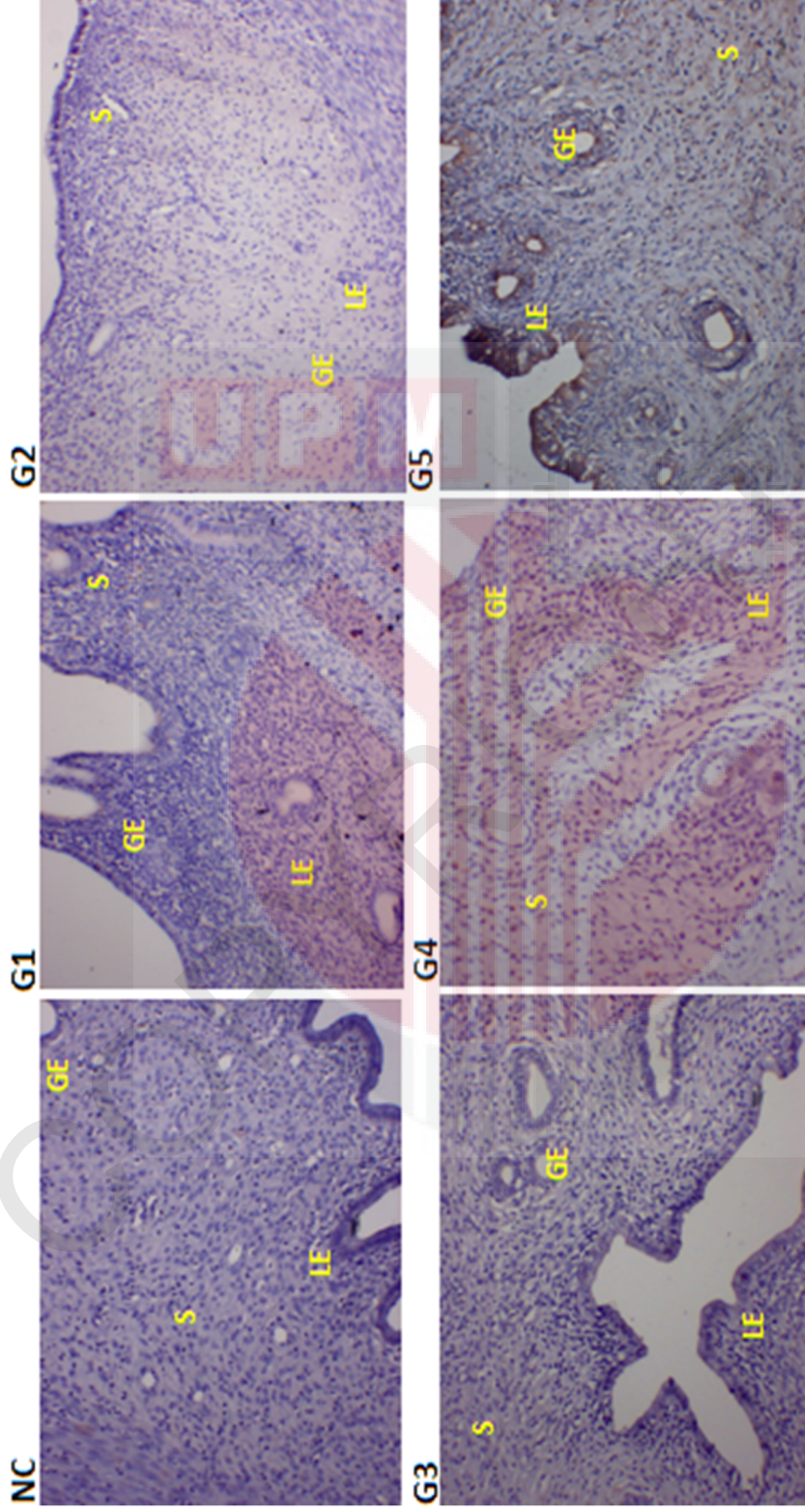


Figure 5.8: EGF expressions in rat endometrial tissues

*NC: without primary antibodies (G1) Control, (G2) Positive control, G2 (IMC + EBN 60 mg/kg bwt), G4 (IMC + EBN 90 mg/kg bwt, G5 (IMC + EBN 120 mg/kg bwt). In LE, EGF immunoreactivity was visible, and staining intensity in the G5 group was relatively higher than in the Control and EBN-treated groups. Magnification 200x.

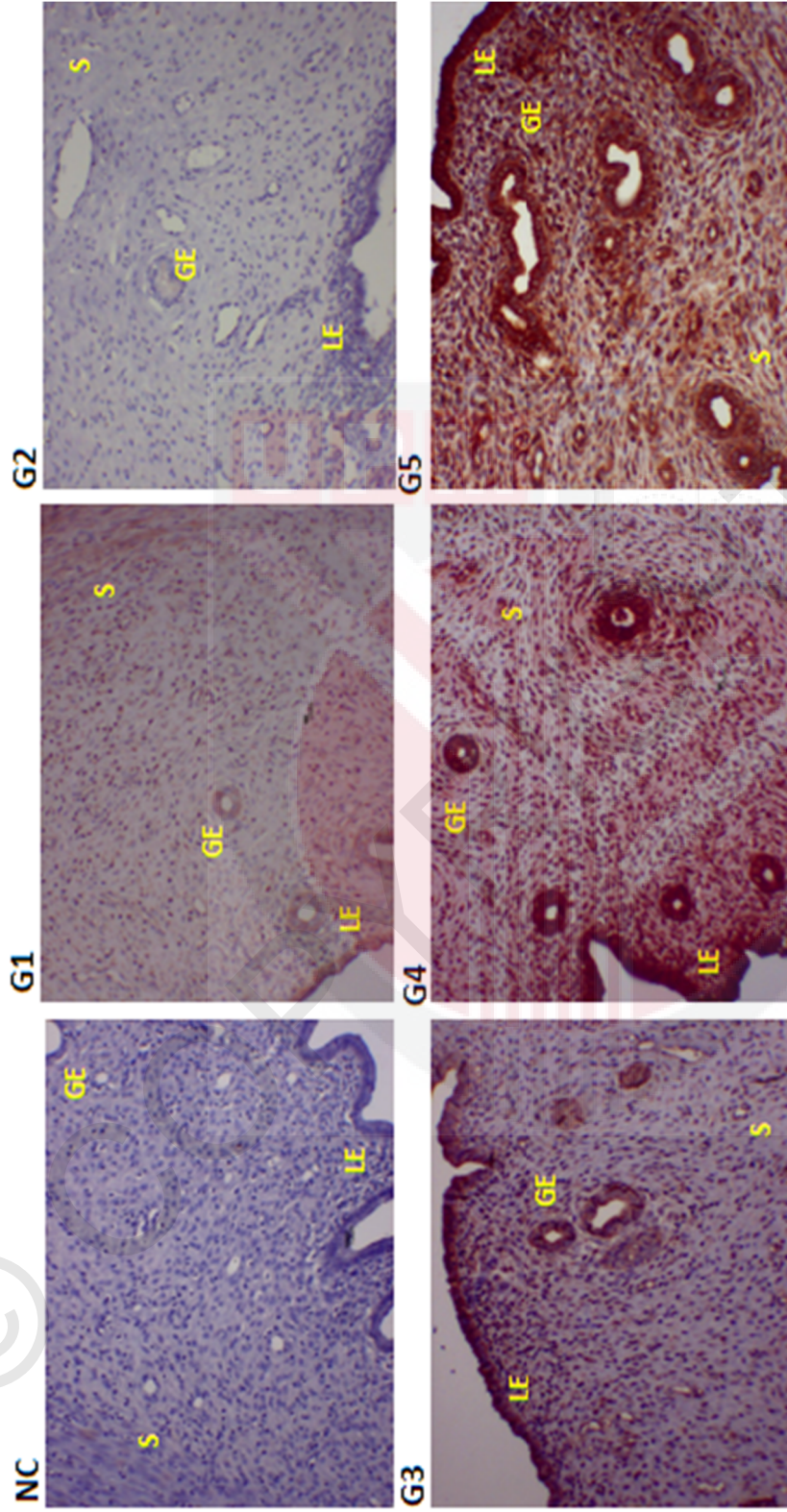


Figure 5.9: EGFR expressions in rat endometrial tissues

*NC: without primary antibodies (G1) Control, (G2) Positive control, G2 (IMC, 4.33 mg/kg bwt), G3 (IMC + EBN 60 mg/kg bwt), G4 (IMC + EBN 90 mg/kg bwt), G5 (IMC + EBN 120 mg/kg bwt). When compared to the Control and EBN treated groups, the intensity of the G5 staining showed greater EGFR immunoreactivity. Magnification 200x.

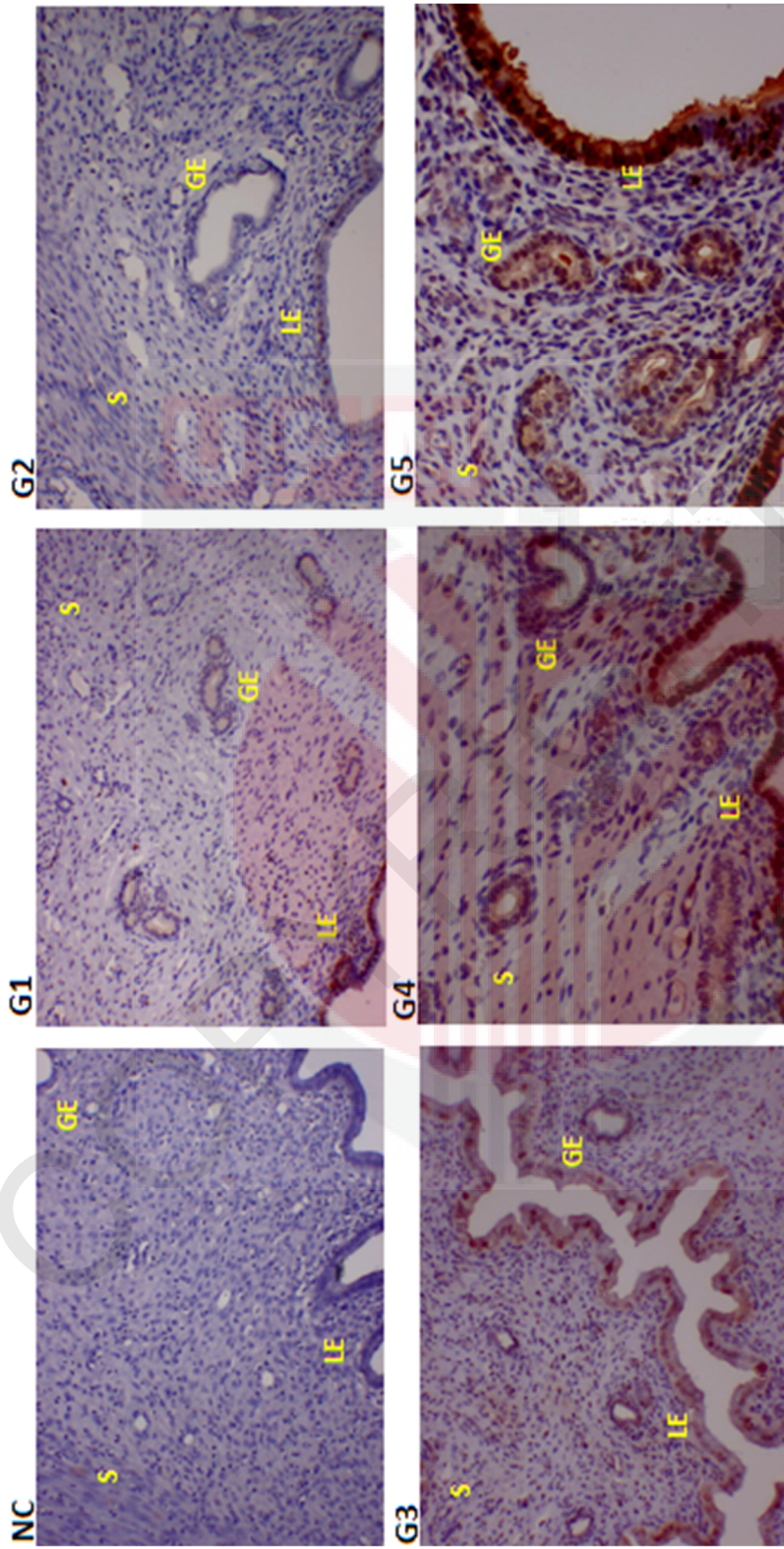


Figure 5.10: PCNA expressions in rat endometrial tissue

*NC: without primary antibodies (G1) Control, (G2) Positive control, G2 (IMC, 4.33 mg/kg bwt), G3 (IMC + EBN 60 mg/kg bwt), G4 (IMC + EBN 90 mg/kg bwt), G5 (IMC + EBN 120 mg/kg bwt). In LE and GE, PCNA immunoreactivity is visible, and the intensity of the staining was increased in the G5 group compared to the Control and EBN-treated groups. Magnification 200x.

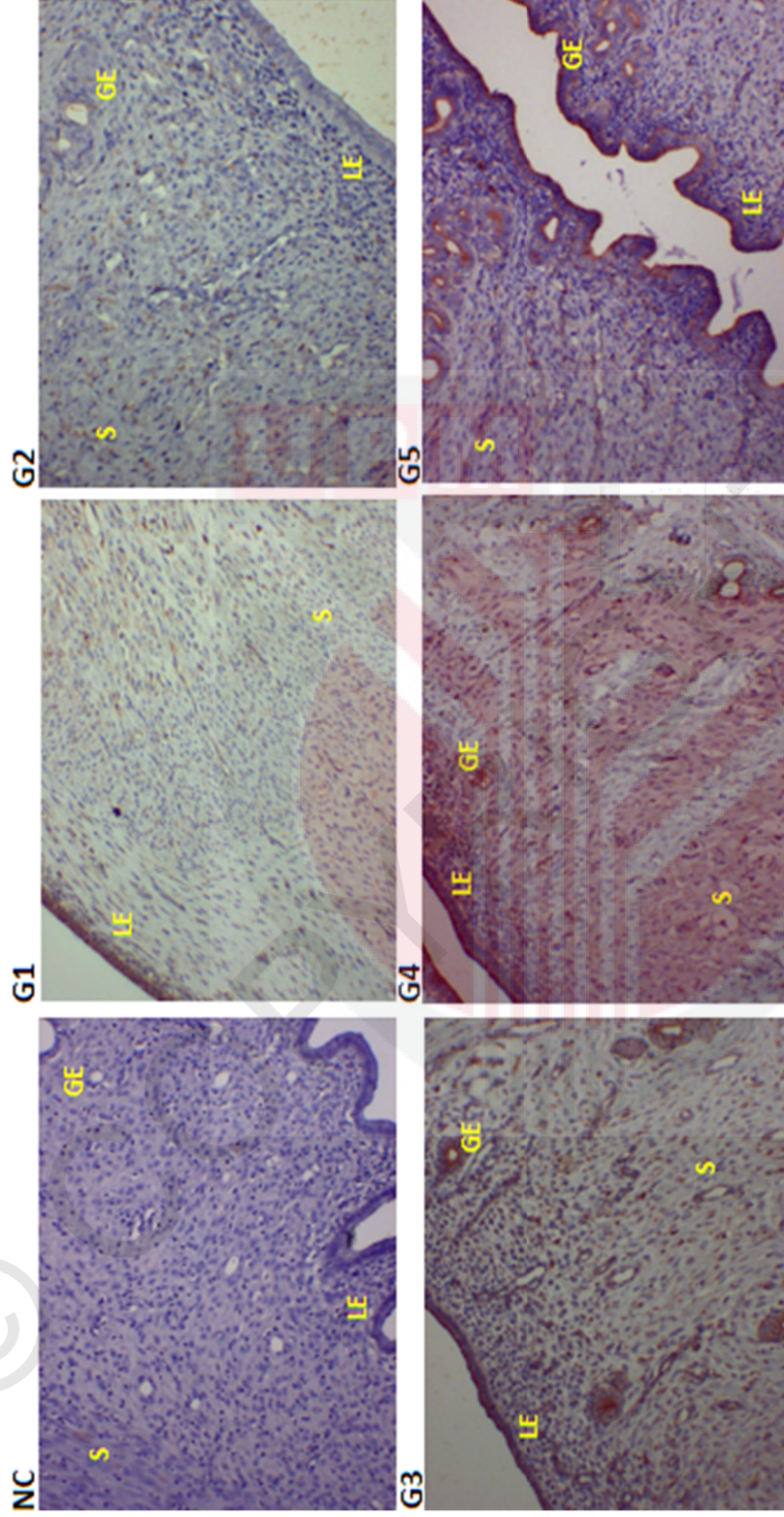


Figure 5.11: VEGF expressions in rat endometrial tissues

*NC: without primary antibodies (G1) Control, (G2) Positive control, G2 (IMC + EBN 60 mg/kg bwt), (E) G4 (IMC + EBN 90 mg/kg bwt), (F) G5 (IMC + EBN 120 mg/kg bwt). The LE exhibits VEGF positivity, and staining intensity in the G5 group was higher than in the Control and EBN-treated groups. Magnification 200x.

Table 5.4: Effect of different levels of edible bird's nest (EBN) fed to indomethacin (IMC)-exposed rats on scores of EGF, EGFR, VEGF and PCNA expression in the uterine tissues (Mean $\pm$ SEM)

Parameters	Groups				
	G1	G2	G3	G4	G5
EGF in LE	1 $\pm$ 0 ^c	0.5 $\pm$ 0 ^c	0.5 $\pm$ 0 ^c	1.5 $\pm$ 0 ^b	3 $\pm$ 0 ^a
VEGF in LE	1.5 $\pm$ 0 ^b	1 $\pm$ 0 ^c	1.5 $\pm$ 0 ^b	3 $\pm$ 0 ^a	3 $\pm$ 0 ^a
EGFR in LE	1 $\pm$ 0 ^c	0.6 $\pm$ 0.3 ^c	1 $\pm$ 0 ^c	1.5 $\pm$ 0 ^b	3 $\pm$ 0 ^a
PCNA in LE	1.3 $\pm$ 0.3 ^b	0.3 $\pm$ 0.16 ^c	1.5 $\pm$ 0 ^b	0.5 $\pm$ 0 ^c	3 $\pm$ 0 ^a
EGF in GE	0 $\pm$ 0 ^c	0 $\pm$ 0 ^c	1.16 $\pm$ 0.16 ^b	2.5 $\pm$ 0 ^b	2.5 $\pm$ 0 ^a
VEGF in GE	1 $\pm$ 0 ^c	0.5 $\pm$ 0 ^c	0.5 $\pm$ 0 ^c	1.5 $\pm$ 0 ^b	3 $\pm$ 0 ^a
EGFR in GE	0 $\pm$ 0 ^c	1 $\pm$ 0 ^c	2 $\pm$ 0 ^b	2.3 $\pm$ 0.6 ^b	2.5 $\pm$ 0 ^a
PCNA in GE	1.3 $\pm$ 1.6 ^b	1 $\pm$ 0 ^c	2.5 $\pm$ 0 ^b	2.5 $\pm$ 0 ^b	2.5 $\pm$ 0 ^a
EGF in S	1.5 $\pm$ 0 ^b	1 $\pm$ 0 ^c	1 $\pm$ 0 ^c	2.5 $\pm$ 0 ^b	3 $\pm$ 0 ^a
VEGF in S	1 $\pm$ 0 ^c	0 $\pm$ 0 ^c	2.5 $\pm$ 0 ^b	2 $\pm$ 0 ^b	2.5 $\pm$ 0 ^a
EGFR in S	0 $\pm$ 0 ^c	0 $\pm$ 0 ^c	0 $\pm$ 0	1 $\pm$ 0 ^c	2.5 $\pm$ 0 ^a
PCNA in S	0 $\pm$ 0 ^c	0.5 $\pm$ 0 ^c	1 $\pm$ 0 ^c	1 $\pm$ 0 ^c	3 $\pm$ 0 ^a

Means with different superscripts within each row differ significantly (P < 0.05)

*G1=Control, G2= (4.33 mg/kg bwt), G3= (4.33+60 mg/kg bwt), G4= (4.33+90 mg/kg bwt), G5= (4.33+120 mg/kg bwt). LE = Luminal epithelium, GE= Glandular epithelium, S= Stroma cell

5.3.7 Expression of steroid hormone ER, and PR in uterine tissues

Representative immunohistochemical photomicrographs of ER and PR expressions in the histological uterine sections of all experimental groups are shown in Figures 5.12 and 5.13, while the corresponding score differences are shown in Table-5.5. The number of stromal cells and the amount of ER expressed in S, GE, and LE all improved as the dose of EBN was raised. The highest-dose EBN-treated rats (G5) showed a statistically and clinically significant (p 0.05) increase in ER expression when compared to the control and other treatment groups (Figure 5.12). Group 2 (G2), the unreactive positive control, showed the second-highest ER expression, which was also considerably greater than that of Group 1 (G1) (Table-5.5). PR immunoreactivity was found in stromal cell and GE nuclei, however. Expressions of PR were modest in the 60 mg/kg bwt (G3) EBN group, highest in the 90 mg/kg bwt (G4) and 120 mg/kg bwt (G5) EBN groups (p<0.05), and lowest in the control (G1) and positive control (G2) groups (Figure 5.13).

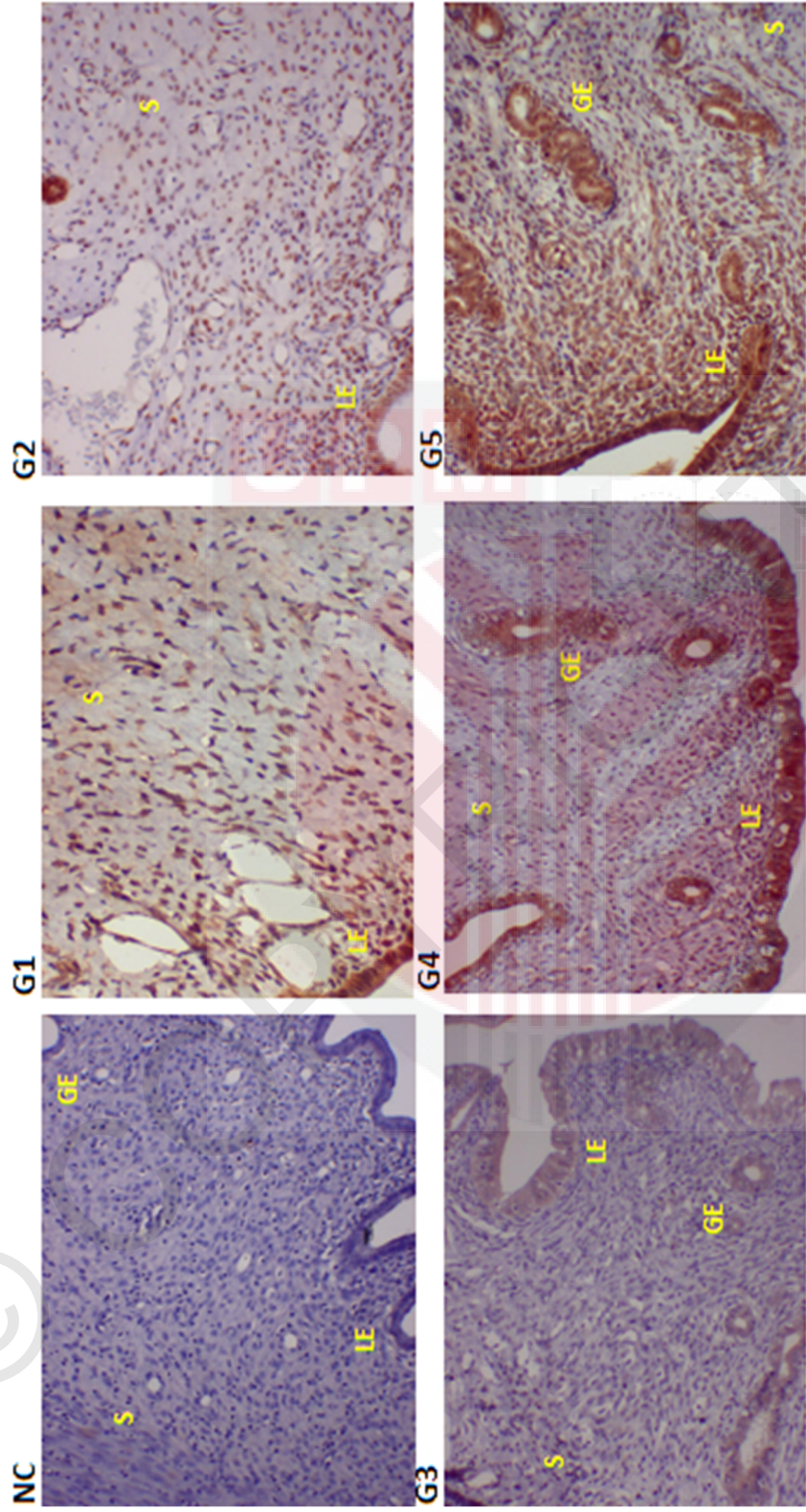


Figure 5.12: ER expressions in rat endometrial tissues

*NC: without primary antibodies (G1) Control, G2 (IMC, 4.33 mg/kg bwt), G3 (IMC + EBN 60 mg/kg bwt), G4 (IMC + EBN 90 mg/kg bwt), G5 (IMC + EBN 120 mg/kg bwt). In LE and GE staining, there is evidence of ER immunoreactivity; G5 staining intensity was higher than in the Control and EBN treated groups. Magnification 200x.

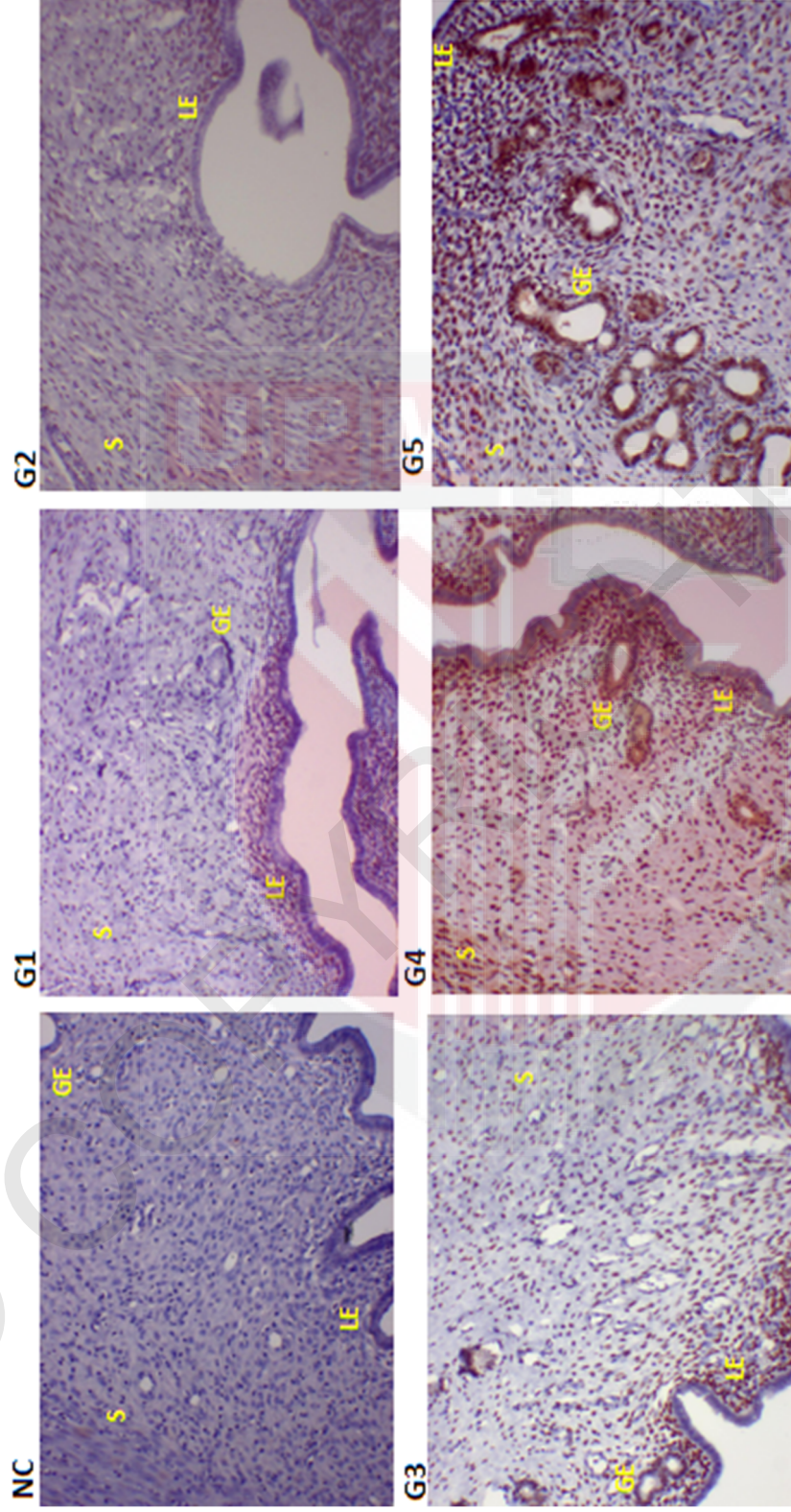


Figure 5.13: PR expressions in rat endometrial tissues

*NC: without primary antibodies (G1) Control, (G2) Positive control, G2 (IMC + EBN 60 mg/kg bwt), (E) G4 (IMC + EBN 90 mg/kg bwt, G5 (IMC + EBN 120 mg/kg bwt). GE displays PR immunoreactivity, and the intensity of the S stain in the G5 group was higher than in the Control and EBN-treated groups. Magnification 200x.

Table 5.5: Effect of different levels of edible bird’s nest (EBN) fed to indomethacin (IMC)-exposed rats on scores for ER and PR expression in uterine tissues (Mean ± SEM)

Parameters	Groups				
	G1	G2	G3	G4	G5
ER in LE	0±0 ^c	0±0 ^c	1.6±0.3 ^b	3±0 ^a	3±0 ^a
ER in GE	1±0 ^c	0±0	2±0 ^a	3±0 ^a	3±0 ^a
ER in S	0.5±0 ^c	0.5±0 ^c	2±0 ^a	2±0 ^a	2.5±0 ^a
PR in LE	0.3±0.16 ^b	0	0±0	2.5±0 ^b	3±0 ^a
PR in GE	0±0	0	2±0 ^a	1±0 ^c	2.8±0.16 ^a
PR in S	1.6±0.16 ^b	2±0 ^a	0.3±0.3 ^c	2.5±0 ^a	3±0 ^a

Means with different superscripts within each row differ significantly (P <0.05).
 *G1=Control, G2= (4.33mg/kg bwt), G3= (4.33+60mg/kg bwt), G4= (4.33+90mg/kg bwt), G5= (4.33+120mg/kg bwt). LE = Luminal epithelium, GE= Glandular epithelium, S= Stroma cells.

5.3.8 Expressions and scoring of EGF, EGFR, PCNA and VEGF in the ovarian tissues

The expressions of epidermal growth factor (EGF), epidermal growth factor receptor (EGFR), PCNA, and vascular endothelial growth factor (VEGF) in ovarian sections from all experimental groups are depicted in detailed immunohistochemical photomicrographs (Figures 5.14–5.17), while Table 5.6 lists the corresponding score differences. In G5 (EBN dose of 120 mg/kg bwt), the histology revealed a dense and larger number of interstitial cells, which also displayed significantly (p < 0.05) higher expression when compared to the positive control (G2) and other treatment groups. This clearly shown that EGF was expressed more highly in G5 (at a dose of 120 mg/kg bwt for EBN). However, when compared to positive and negative control and other EBN treated groups, the EGF expression level revealed a considerably (p < 0.05) strong staining reaction as shown in the 120mg/kg bwt (G5) of the EBN group (Figure-5.14). Moreover, G5 (EBN dose of 120 mg/kg bwt) demonstrated a dense and higher number of interstitial cells in comparison to the positive control (G2) and the remaining treatment groups (Figure-5.15- 5.17). In contrast, EGFR and PCNA reactions began to be noticed in group G4 at 90 mg/kg bwt of REBN supplement with a comparable level of expression and staining as compared to a dose of 120 mg/kg bwt

of EBN supplement ($p > 0.05$). Yet only G5 of the EBN group, at 120 mg/kg bwt, displayed the greater expression (Figure-5.15- 5.16). Nevertheless, VEGF effects became evident at a supplement dose of 60 mg/kg bwt of EBN (G3). In Figure 5.17, the VEGF control (G1) and positive control (G2) groups appeared to exhibit some fairly tepid replies. However substantially ($p > 0.05$), the EBN group's 120 mg/kg bwt (G5) VEGF showed the highest expression.



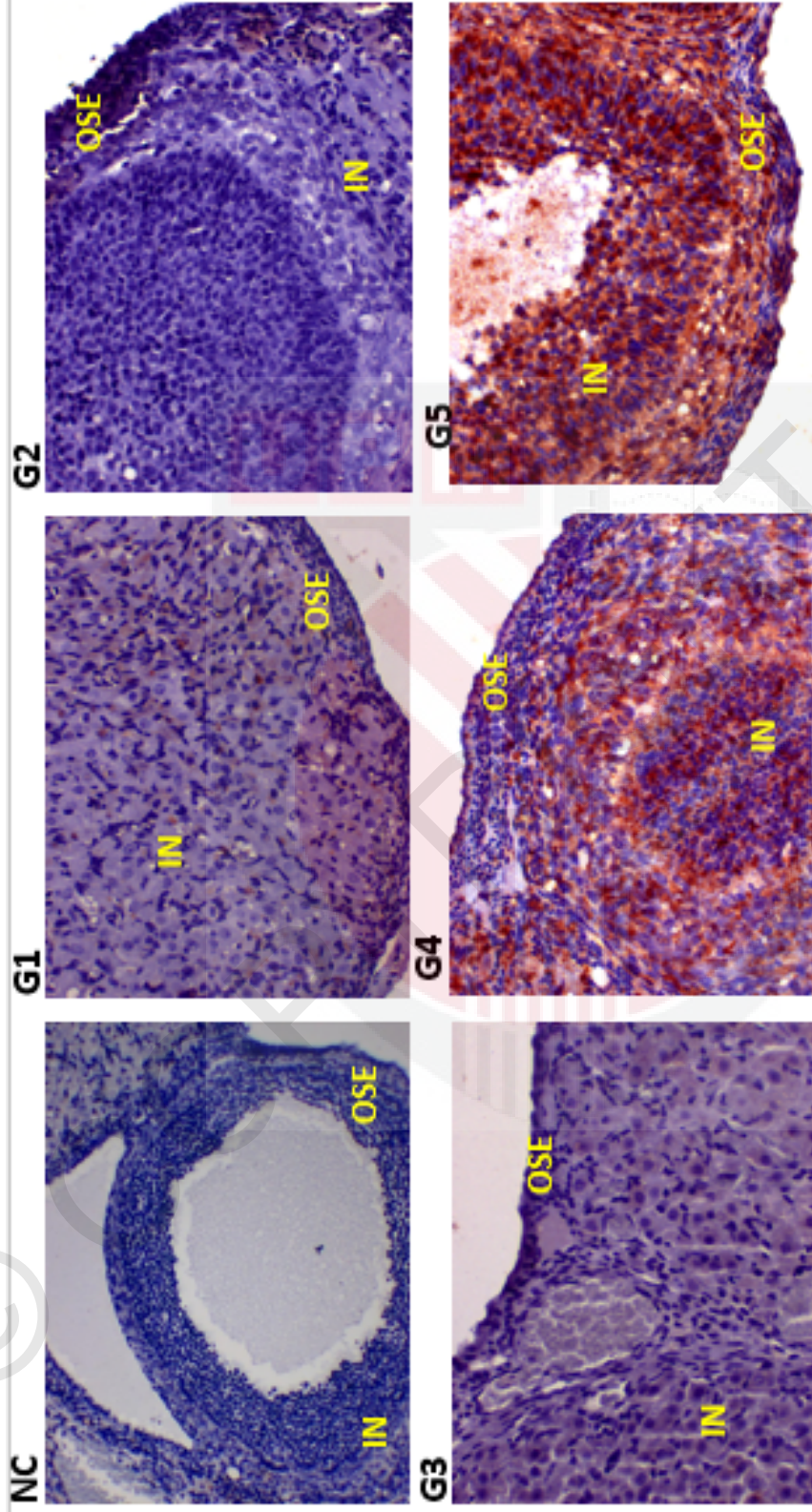


Figure 5.14: EGF expressions in rat ovarian tissues

*NC: without primary antibodies (G1) Control, (G2) Positive control, G2 (IMC + EBN 60 mg/kg bwt), G4 (IMC + EBN 90 mg/kg bwt), G5 (IMC + EBN 120 mg/kg bwt). Compared to G2 and other treatment groups, interstitial cells were more numerous and denser in the G5 cell line. It shows EGF; higher expressions noticed in G5. OSE= Ovarian surface epithelium, IN= interstitial cells. Magnification 200x.

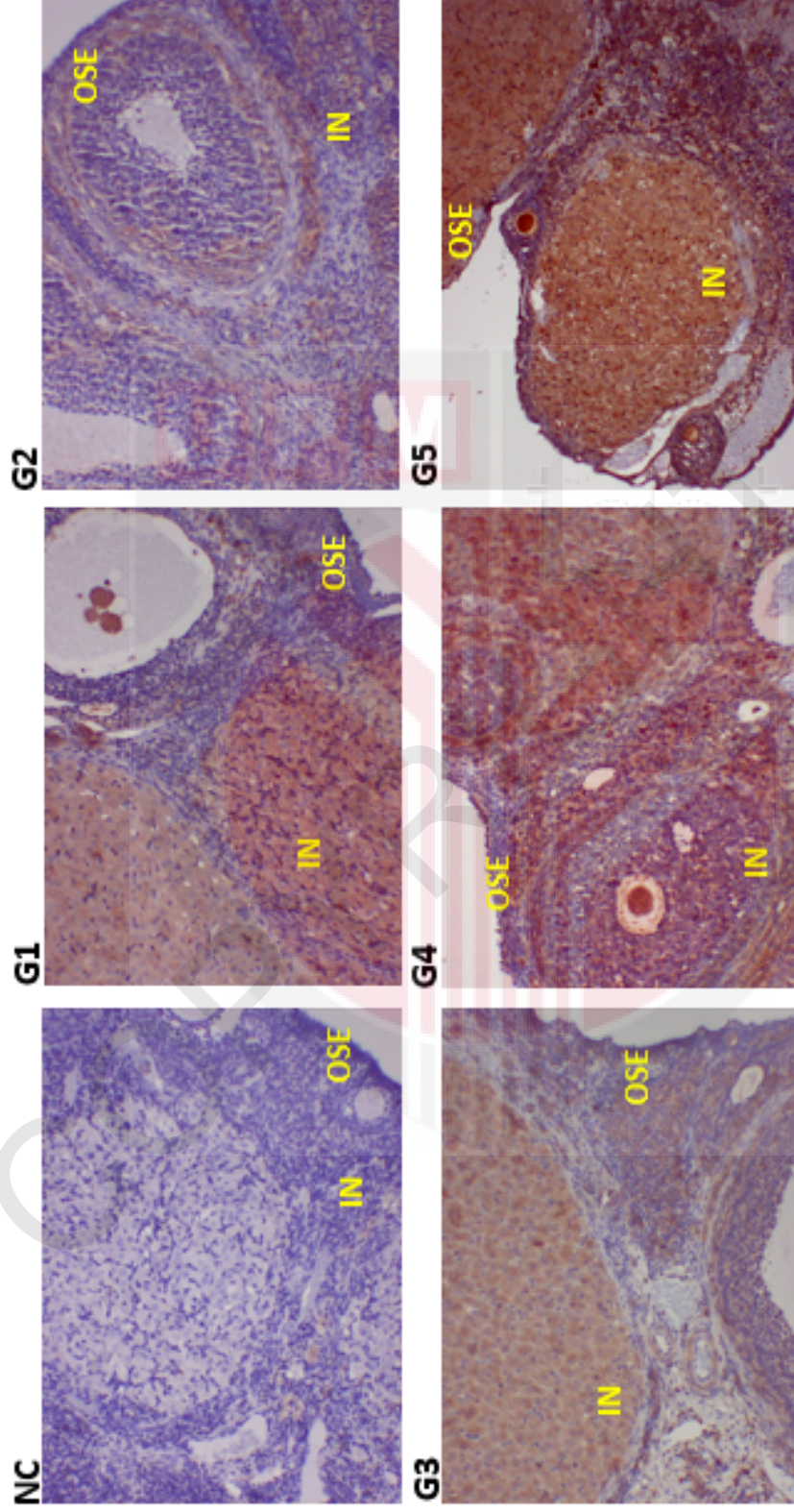


Figure 5.15: EGFR expressions in rat ovarian tissues

*NC: without primary antibodies (G1) Control, (G2) Positive control, G2 (IMC + EBN 60 mg/kg bwt), G4 (IMC + EBN 90 mg/kg bwt, G5 (IMC + EBN 120 mg/kg bwt). Compared to G2 and other treatment groups, interstitial cells were more numerous and denser in the G5 cell line. It shows EGFR; higher expressions are visible in G5. OSE= Ovarian surface epithelium, IN= interstitial cells. Magnification 200x.

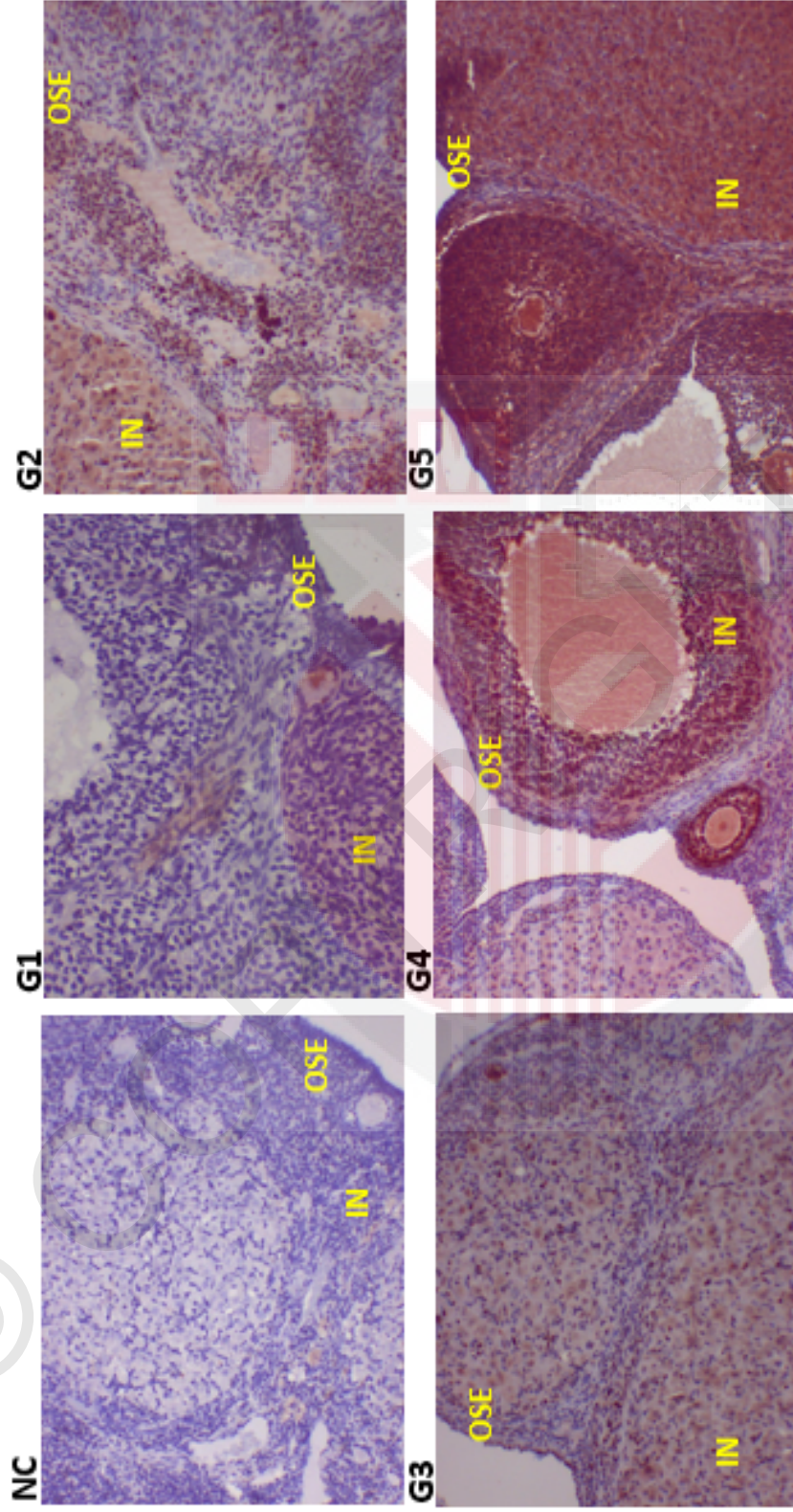


Figure 5.16: PCNA expressions in rat ovarian tissues

*NC: without primary antibodies (G1) Control, (G2) Positive control, G2 (IMC, 4.33 mg/kg bwt), G3 (IMC + EBN 60 mg/kg bwt), G4 (IMC + EBN 90 mg/kg bwt, G5 (IMC + EBN 120 mg/kg bwt). G5 interstitial cells are more abundant and denser than G2 and the other treatment groups. It shows PCNA; remarkably higher expressions are visible in G5. OSE= Ovarian surface epithelium, IN= interstitial cells. Magnification 200x.

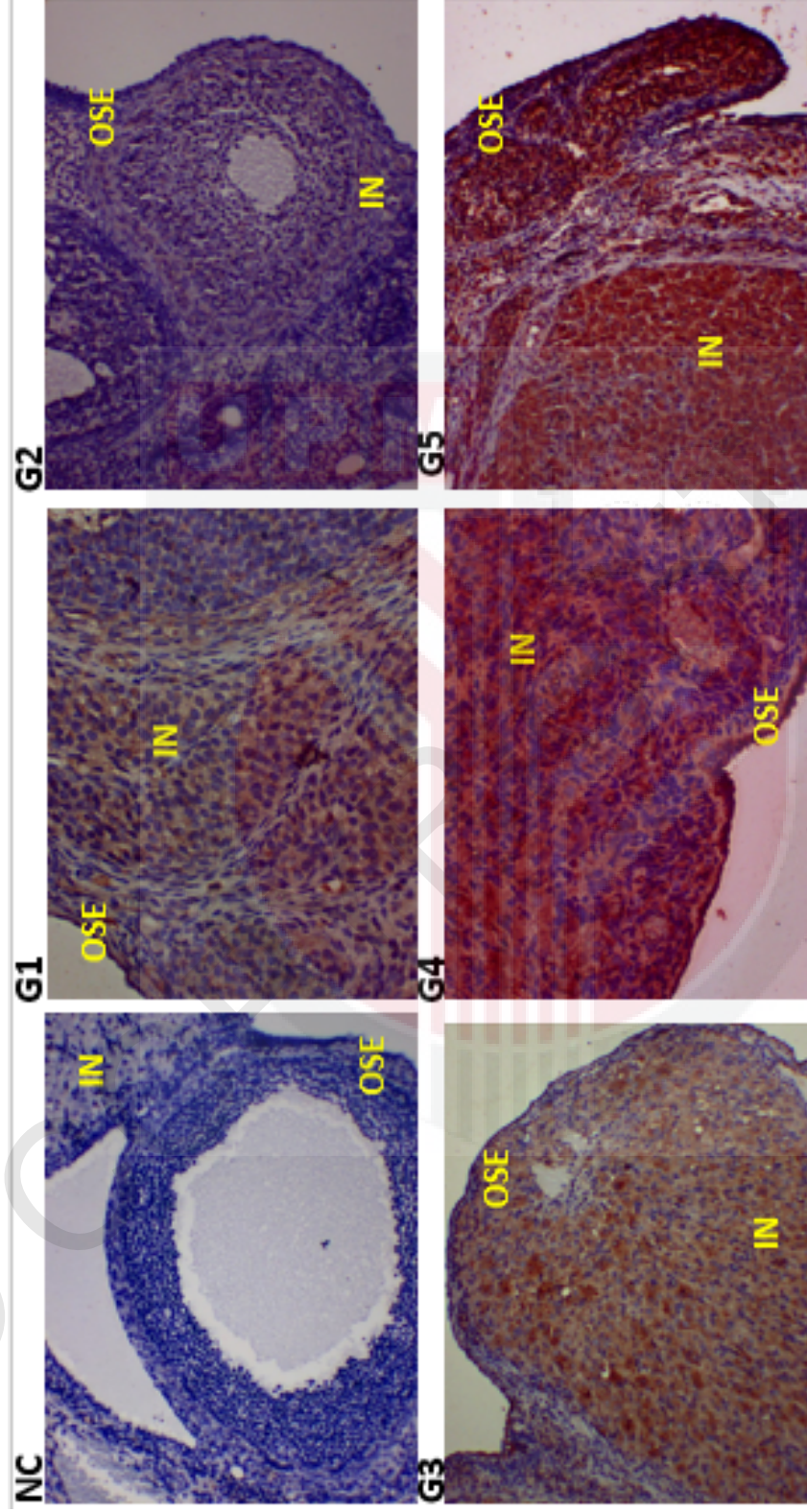


Figure 5.17: VEGF expressions in rat ovarian tissues

*NC: without primary antibodies (G1) Control, (G2) Positive control, G2 (IMC + EBN 60 mg/kg bwt), G4 (IMC + EBN 90 mg/kg bwt), G5 (IMC + EBN 120 mg/kg bwt). Interstitial cells in G5 are more numerous and denser than in other treatment groups. It shows VEGF; remarkably higher expressions are visible in G5. OSE= Ovarian surface epithelium, IN= interstitial cells. Magnification 200x.

Table 5.6: Rats given EBN supplements and subjected to IMC toxicity displayed average levels of EGF, EGFR, VEGF, and PCNA expression in their ovarian tissues

Parameters	Groups				
	G1	G2	G3	G4	G5
EGF in OSE	1±0 ^c	0.5±0 ^c	0.5±0 ^c	1.5±0 ^b	3±0 ^a
EGF in interstitial cells	1±0 ^c	0.5±0 ^c	0.5±0 ^c	1.5±0 ^b	3±0 ^a
VEGF in OSE	0±0	0±0	1.16±0.16 ^b	2.5±0 ^a	2.5±0 ^a
VEGF in interstitial cells	1±0 ^c	0±0	2.5±0 ^a	2±0 ^a	2.5±0 ^a
EGFR in OSE	1.5±0 ^b	1±0 ^c	1±0 ^c	2.5±0 ^a	3±0 ^a
EGFR in interstitial cells	1±0 ^c	0.6±0.3 ^c	1±0 ^c	1.5±0 ^b	3±0 ^a
PCNA in OSE	1.5±0 ^b	1±0 ^c	1.5±0 ^b	3±0 ^a	3±0 ^a
PCNA in interstitial cells	0±0	1±0 ^c	2±0 ^a	2.3±0.6 ^a	2.5±0 ^a

Within the same row are means with different superscripts significantly different from each other at p<0.05. *G1=Control, G2= (4.33mg/kg bwt), G3= (4.33+60mg/kg bwt), G4= (4.33+90mg/kg bwt), G5= (4.33+120mg/kg bwt). OSE = Ovarian surface epithelium, interstitial cells.

5.3.9 The expression of steroid hormone receptors, ER and PR in the ovarian tissues

Immunohistochemical photomicrographs of representative uterine sections from all experimental groups showing ER and PR expressions are shown in Figures 5.18 and 5.19, respectively, and Table 5.7 lists the corresponding score differences. EBN upregulated ER expression and interstitial cell density in the ovary. Maximum-dose EBN-treated rats, 120 mg/kg, were compared to both untreated and other treated groups bwt (G5)—exhibited a substantial ($p < 0.05$) rise in ER expression (Figure 5.18). On the other hand, the EBN group's 120 mg/kg bwt (G5) demonstrated the highest expression of PR immunoreactivity. As can be seen in (Figure 5.19), the positive control group (G2) had the lowest expressions compared to both the negative control group and the EBN-treated groups.

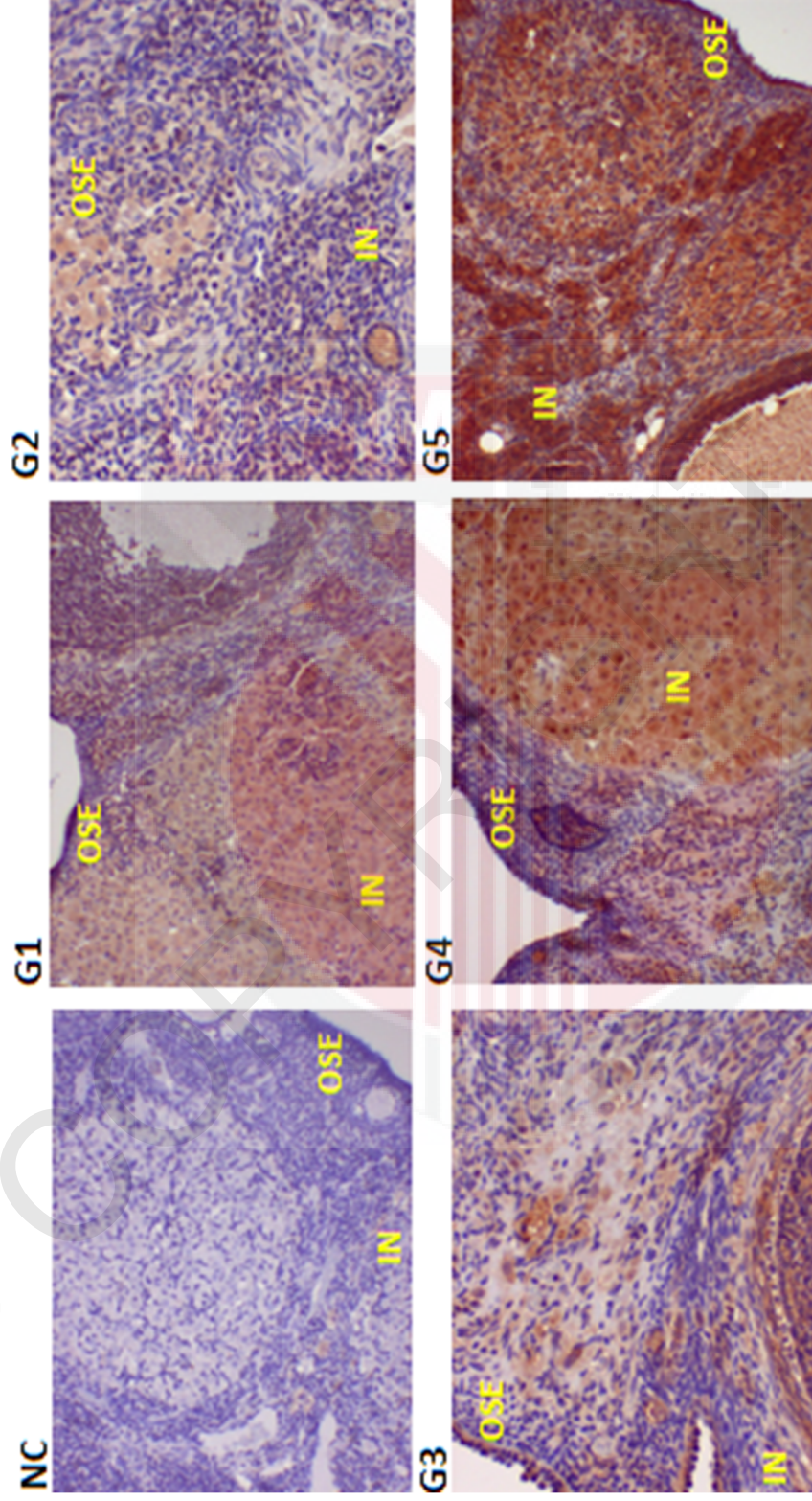


Figure 5.18: ER expressions in rat ovarian tissues

*NC: without primary antibodies (G1) Control, (G2) Positive control, G2 (IMC + EBN 60 mg/kg bwt), G4 (IMC + EBN 90 mg/kg bwt, G5 (IMC + EBN 120 mg/kg bwt). Compared to G2 and other treatment groups, interstitial cells were more numerous and denser in the G5 cell line. It shows E2R; higher expressions are visible in G5. OSE= Ovarian surface epithelium, IN= interstitial cells. Magnification 200x.

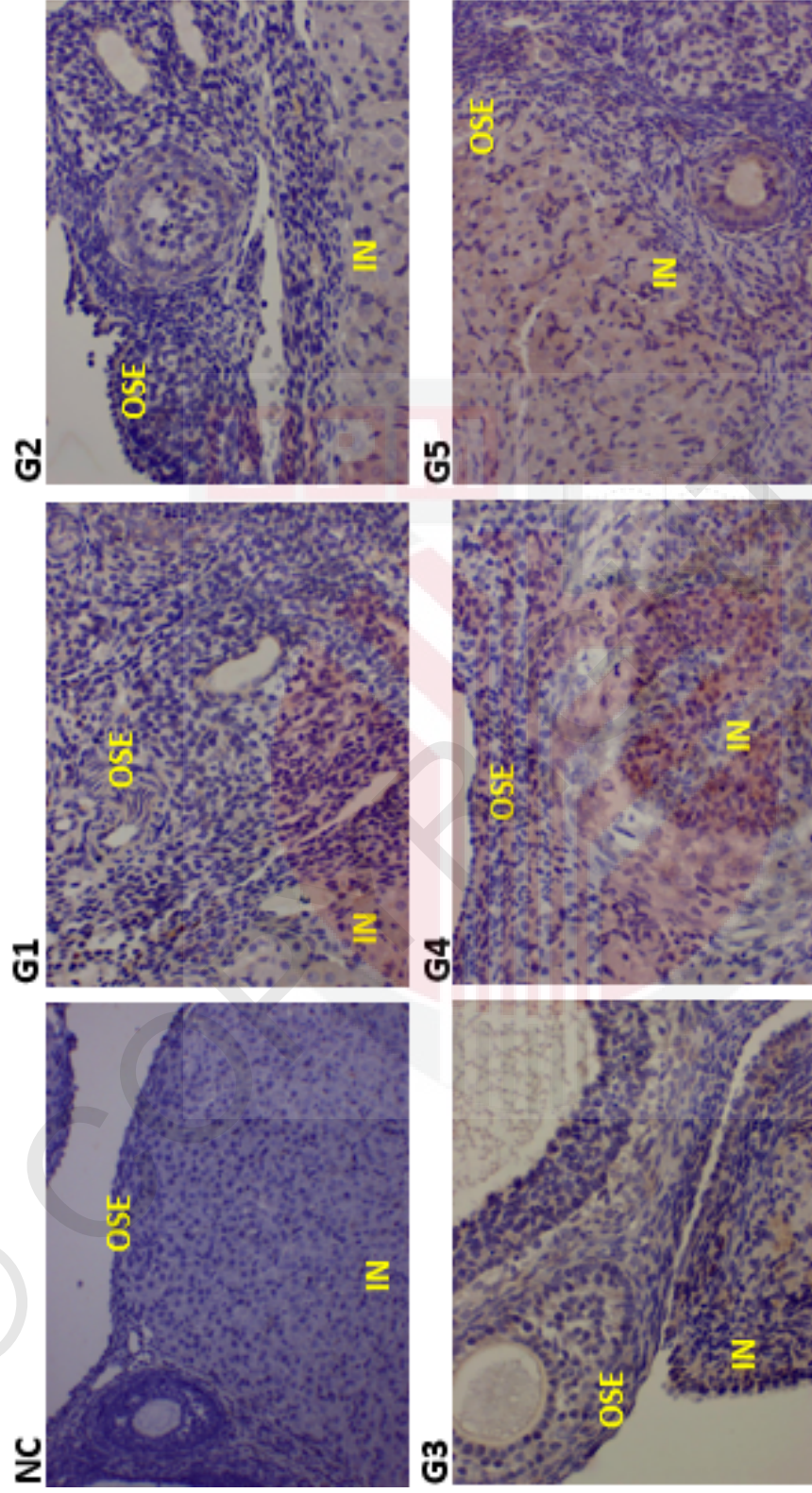


Figure 5.19: PR expressions in rat ovarian tissues

*NC: without primary antibodies (G1) Control, (G2) Positive control, G2 (IMC + EBN 90 mg/kg bwt, G5 (IMC + EBN 120 mg/kg bwt). Compared to G2 and other treatment groups, interstitial cells were more numerous and denser in the G5 cell line. It shows P4R; higher expressions are visible in G5. OSE= Ovarian surface epithelium, IN= interstitial cells. Magnification 200x.

Table 5.7: Rats given EBN supplements and exposed to IMC poisoning displayed average levels of ER and PR expression in their ovarian tissues

Parameters	Groups				
	G1	G2	G3	G4	G5
ER in OSE	0±0 ^c	0±0	1.6±0.3 ^b	3±0 ^a	3±0 ^a
ER in interstitial cells	1±0 ^b	0±0	2±0 ^a	3±0 ^a	3±0 ^a
PR in OSE	0.5±0 ^c	0.5±0 ^c	2±0 ^a	2±0 ^a	2.5±0 ^a
PR in interstitial cells	0.5±0 ^c	0±0	0±0	2.5±0 ^a	3±0 ^a

Within the same row are means with different superscripts significantly different from each other at p<0.05. *G1=Control, G2= (4.33mg/kg bwt), G3= (4.33+60mg/kg bwt), G4= (4.33+90mg/kg bwt), G5= (4.33+120mg/kg bwt). OSE = Ovarian surface epithelium, interstitial cells.

5.3.10 Influence of EBN on the SOD and TBARS levels

In comparison to the treatment groups and control, TBARS were reduced in the 120 mg/kg bwt (G5) of the EBN group and higher (p<0.05) in the positive control (G2) group. The alterations reached the maximum value (p<0.05) in G2 and dramatically decreased in the therapy groups (Figure 5.20).

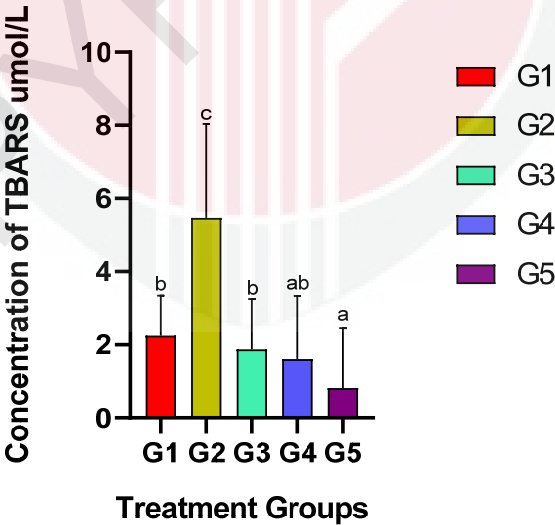


Figure 5.20: Plasma concentration of TBARS of treatment groups when compared to control and IMC group. TBARS = thiobarbituric acid reactive substance

Data are expressed as Mean±SEM. Different letters a, b and c within rows denotes significant difference at p < 0.05. Note: G1=Control, G2= (IMC 4.33mg/kg bwt), G3= (IMC 4.33mg/kg, EBN 60mg/kg bwt) G4= (IMC 4.33mg/kg, EBN 90mg/kg bwt) and G5= (IMC 4.33mg/kg, EBN 120mg/kg bwt).

SOD was lower in the 60mg/kg bwt (G3) of the EBN group and higher ($p<0.05$) in the 120mg/kg bwt (G5) of the EBN group in compared with the control and treatment groups. In the treatment groups, SOD significantly increased ($p<0.05$) value in G5 of the EBN group (Figure 5.21).

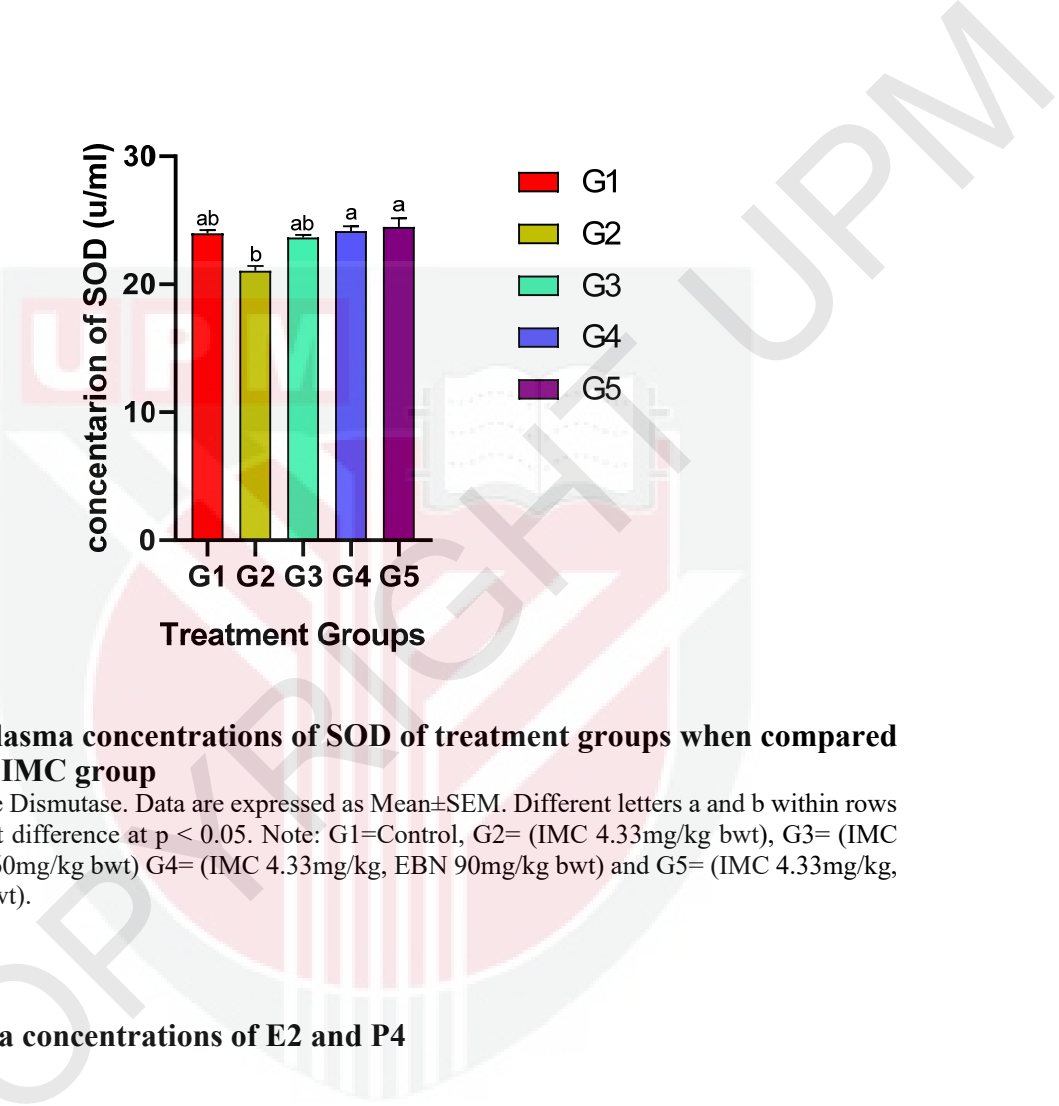


Figure 5.21: Plasma concentrations of SOD of treatment groups when compared to control and IMC group

SOD = Superoxide Dismutase. Data are expressed as Mean±SEM. Different letters a and b within rows denotes significant difference at $p < 0.05$. Note: G1=Control, G2= (IMC 4.33mg/kg bwt), G3= (IMC 4.33mg/kg, EBN 60mg/kg bwt) G4= (IMC 4.33mg/kg, EBN 90mg/kg bwt) and G5= (IMC 4.33mg/kg, EBN 120mg/kg bwt).

5.3.11 Plasma concentrations of E2 and P4

Oestrogen was lower in the 60mg/kg bwt (G3) of the EBN group and higher ($p<0.05$) in the 120mg/kg bwt (G5) of the EBN group as compared to the control (G1) and treatment groups. In the treatment groups, the modifications considerably increased and attained the greatest value ($p<0.05$) in the 120mg/kg bwt (G5) of the EBN group (Figure 5.22A). Similarly, the progesterone was lesser in positive control group (G2) and greater ($p<0.05$) in 120mg/kg bwt (G5) of the EBN group while comparing it to

control (G1) and treatment groups. In the treatment groups, the modifications considerably increased and attained the greatest value ($p<0.05$) in 120mg/kg bwt (G5) of the EBN group (Figure 5.22B).

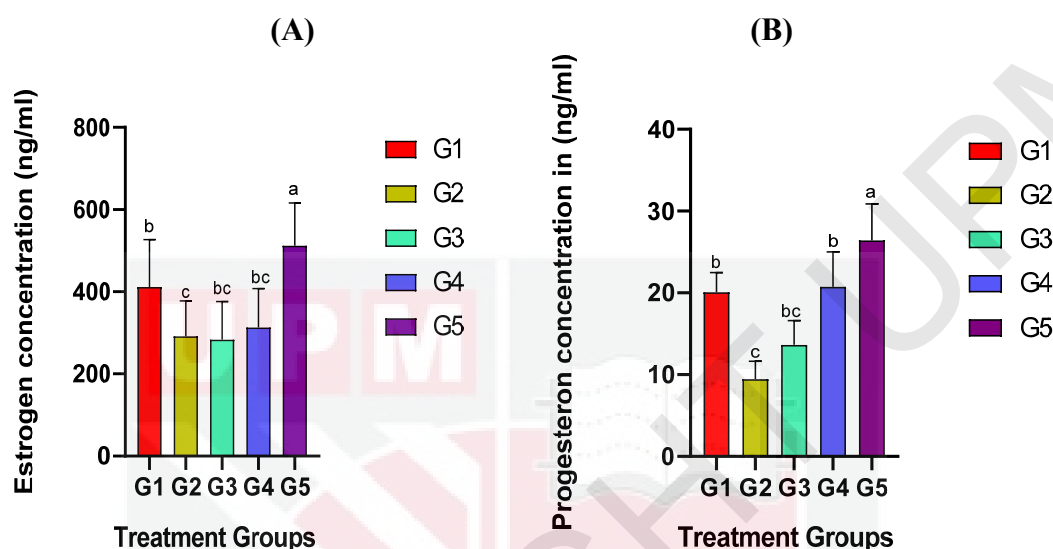


Figure 5.22: Plasma concentration of oestrogen (A) and progesterone (B) hormones as influenced by edible bird's nest (EBN) supplementation or female rats exposed to indomethacin (IMC)

Data are expressed as $M \pm SEM$. Error bars show different letters a, b, c denotes significant difference at $p<0.05$. *G1=Control, G2= (4.33mg/kg bwt), G3= (4.33+60mg/kg bwt), G4= (4.33+90mg/kg bwt), G5= (4.33+120mg/kg bwt)

5.4 Discussion

A tremendous attempt to find effective anti-inflammatory and analgesic drugs resulted in the discovery of IMC, an extraordinarily strong NSAID (Lucas & Pain, 2016). The cyclooxygenase inhibitors they work as, whether nonselective or selective, may have more potent vasoconstrictive effects, but IMC may also have unique direct neuronal inhibitory pathways or nitric oxide-dependent inhibitory pathways. The majority of NSAIDs have been found to have a detrimental effect on PG production by inhibiting COX1 and COX2 enzymatic activity (Dannhardt et al., 2001; Bhunyakarnjanarat et al., 2021). However, it has been proposed that the re-released of IMC into the general

circulation can be triggered by a variety of circumstances (Dimick et al., 2021). As a result, IMC has intermediate half-life (4.5+ hours) (Villar et al., 2021). Previous research has typically found that IMC has a negative impact on mature female reproductive organs and fertility (Frungieri et al., 2021). Despite being exposed to IMC, female rats' oestrous cycles were normal, according to histological results from the current study. IMC administered at different doses were shown to cause bwt and uterine weight reduction, along with a frequency of the pregnancy within mice and implanted ova were also reported to be decreased, by (Csányi & Ducza, 2019). There have been various studies and research reported on the biological features and chemical composition of EBN, but there have not been studies on effects of EBN on uterine damage induced by IMC. The UBWR and BMI were both reduced due to IMC. Epithelium layer cells that were destroyed and a reduction in the number of endometrial glands investigated by histological examination were shown to be associated with the thickness of the uterine lumen, atrophy of the endometrium, vacuolar degeneration, inflammatory alterations, and muscle atrophy. EBN supplemented groups (especially treated with 120mg/kg bwt of the EBN group) demonstrated greater levels of standard uterine structures and uterine morphological parameters despite exposure to IMC toxicology, in accordance with (Eugenia et al., 2009). Prior research found a percent change of 135 to 215 in the human colonic cancer cell line Caco-2 when compared to the control by edible bird's nest, and that it's decreased the percentage of tumour necrosis factor-alpha (TNF-) (Aswir & Wan Nazaimoon, 2011; Vimala et al., 2012). Similarly anti-inflammatory characteristics may exist within the EBN extract because it inhibits the generation of nitric oxide, according to the scientists (Vimala et al., 2012), as substantiated by (Zainal Abidin et al., 2011). New Zealand white rabbit corneal keratocytes were produced and cultured

in both serum-containing and serum-free medium, can benefit from a modest concentration of EBN (0.05 percent or 0.1 percent). In the EBN extract, the presence of EGF was found to be a contributing factor to the enhanced proliferation. Edible bird's nest's wound healing qualities have been hailed as a breakthrough in the field of corneal wound healing. According to (Wong et al., 2012), It has been demonstrated that various Chinese herbal medications, including EBN, promote adult stem cell development during tissue regeneration. The edible bird's nest group at 120 mg/kg bwt (G5), however, significantly developed and produced more endometrial structures in the uterus, according to the current study. On the other hand, the ovary's histological examination revealed ovarian tissue atrophy in the groups of edible bird's nest that received the positive control (G2), 60 mg/kg bwt (G3), and 90 mg/kg bwt (G4) doses.

EBN inhibits embryonic neuronal apoptosis by interfering with caspase-3 activation, resulting in the suppression of apoptotic cells (Jin et al., 2001). Testosterone, E2, P4, LH, FSH and prolactin have been found to be present in EBN in a previous investigation into its composition . In a recent study, it was found that VEGF, FSH, and oestradiol work together to prevent apoptosis, block the caspase 3 activation, and to promote proliferation (Vitale et al., 2002; Busmann et al., 2006; Irusta et al., 2010). EBN's ability to affect uterus may be due to the presence of bioactive chemicals. Protein, carbohydrates, ash, and lipids make up the majority of a food's nutritional value . Edible bird's nest's water-soluble components, including proteins, carbohydrates, iron, inorganic salts, and fibre, make it an excellent source of nutrients. The chemical pathways that drive cell proliferation in sialic acid are similar to those in EBN. The human endometrium expresses a sialic acid binding protein that is controlled by the oestrogen hormone oestradiol. Edible bird's nest's EGF-like

component has been reported to linked to its part within the process of cell division, proliferation, and regeneration of tissue. EBN's rejuvenating properties are attributed to this phenomenon (Bussmann et al., 2006; Ma et al 2012). The current study found that the level of EGF expression of uterus went from being low within control group (G1) and in group that got low dose of EBN (G3) to the one being medium in 90 mg/kg bwt (G4) and to the high dose of 120 mg/kg bwt (G5) groups. Ovary clearly expressed EGF, with a higher level of expression in G5 (120 mg/kg bwt of EBN).

According to the findings of this study, the IMC's restriction of PGs is the cause of the vasoconstriction in uterine tissues. It's still consistent with the findings of (Ekanem et al., 2008). Aside from ischaemia, smooth muscle atrophy can be a direct result of a loss of blood flow to the muscle (Bishop-Bailey & Warner, 2003). Nevertheless, the results of this study show that IMC formation caused by PG inhibition results in uterine muscle atrophy, which is hazardous. High doses of a NSAID have been shown to alter the walls of uterine horns and lower the capacity of the lumen and the layers of the lamina propria and the epithelium (Güven et al., 2013). Similarly, the treatment of IMC drastically reduced uterine horn and ancillary function shrinkage, as well as ciliary activity and uterine horn lumen. After renal excision, increased PG renal excretion is the primary cause of elevated COX-2 expression (Sanchez et al., 1999). It was shown that when rats exposed to IMC were given edible bird's nest, the cells and glands in their uterine tissues were more active. IMC may harm uterine glands, altering endometrial glandular secretions of enzymes, growth factors, cytokines, hormones, and transport proteins crucial for the conceptus' development, which may have a negative impact on in-utero physiological processes (Spencer & Bazer, 2004). A measure of DNA synthesis and cell proliferation, PCNA density increased as well. These results provide more proof that edible bird's nest may have a role in fertility and

reproduction. EGF, VEGF, and interleukin-6 are intercellular mediators that influence cellular differentiation, survival, and development in the body. They are essential for cellular functioning (Hirano et al., 2000; Alan et al., 2015). Our most recent research, which demonstrates a relationship between high levels of EGF expression and increased ovarian and uterine tissue proliferation as well as endothelium thickness, lends credence to this concept. It has been demonstrated in the past that edible bird's nest increases the growth factors EGF, VEGF, and PCNA in uterine cells, causing the cells to proliferate. As a result, the presence of VEGF in the endometrium is necessary for structural growth, maintenance, and breakdown. Also, it's critical to remember that VEGF is essential for promoting blood flow to the uterine glands and stromal cells. Supplementing with edible bird's nest promotes the development of LE and GE stromal cells when VEGF levels are high. VEGF and luteinizing hormone, two substances released by luteinizing cells, are related (Albishtue et al., 2018). EGFR, PCNA, and VEGF expression was also shown to be elevated in the present study, along with stromal cells, glandular and uterine epithelium. Since EGF, EGFR, VEGF, and PCNA were all shown to be upregulated after ingestion of bird's nest, it's possible that this food has a proliferative effect as well as therapeutic benefits against IMC toxicity (Kinnaroy et al., 2014).

Nitrogen oxides and reactive oxygen species kill cells. Respiratory burst and cellular damage are brought on by superoxide and hydrogen peroxide. Malondialdehyde, glutathione, catalase, superoxide dismutase, reactive oxygen species, nitric oxide synthase, and indicators of lipid peroxidation all have connections to various disorders. The antioxidants in bird's nest, which can be eaten, have medicinal and preventative uses (Albishtue et al., 2019). A good source of natural antioxidants and potential nutraceutical or pharmaceutical compound, bird's nest is edible. In the Parkinson's disease model SH-SY5Y cells, it guards against oxidative damage Hou et al. 2015)

proved that the brain is shielded against ageing brought on by a loss of oestrogen by the consumption of bird's nest (Hou et al., 2015). The levels of SOD and TBARS were measured in the current study. SOD was significantly increased in the G5 group. The positive control group's TBAR concentration was discovered to be significantly greater than that of the control and treatment groups.

5.5 Conclusion

Supplementation of female rats with edible bird's nest (EBN) may mitigate the reproductive toxicity induced by IMC therapy and exhibit significant effects on reproductive performance. Among the groups receiving both IMC and EBN, the highest number of implantation sites was observed at a dosage of 120 mg/kg. In the ovarian and uterine tissues, EBN demonstrated solid antioxidant properties, promoting tissue proliferation by enhancing levels of Superoxide Dismutase (SOD) and upregulating the expression of Epidermal Growth Factor (EGF), Epidermal Growth Factor Receptor (EGFR), Vascular Endothelial Growth Factor (VEGF), Proliferating Cell Nuclear Antigen (PCNA), and steroid hormones. The findings of this study indicate that IMC treatment in Sprague-Dawley (SD) rats hindered embryo implantation and growth. However, a dosage of 4.33 mg/kg bwt of IMC was sufficient to induce Experimental Induced Dysfunction (EID) while causing less harm to vital organs compared to higher dosages. Animals administered EBN at a level of 120 mg/kg bwt exhibited remarkable protective effects against IMC-induced toxicity on fertility and reproductive performance, thus highlighting the role of edible bird's nest in mitigating the impact of IMC on the uterus and ovaries with increasing dosage.

CHAPTER 6

COMPARATIVE ASSESMENT OF THE PROTECTIVE EFFECT OF RAW AND HYDROLYZED EBN AGAINST LEAD ACETATE TOXICITY ON RAT'S REPRODUCTIVE SYSTEM HISTOMORPHOLOGY, EMBRYO IMPLANTATION, AND HORMONE PROFILE

6.1 Introduction

Lead acetate is a hazardous metal that has been shown to have negative effects on several body systems, including reproduction (Mokhtari & Zanoori, 2011). The possible risks of lead exposure to reproductive health have prompted the investigation of preventative options (Kumar, 2018). The saliva of cave-dwelling swiftlets is the source of edible bird's nest (EBN), a unique natural substance with a variety of therapeutic applications (Chua & Zukefli, 2016). EBN is a good candidate for reducing the harmful effects of lead acetate on the reproductive system due to its high protein, amino acid, mineral, and bioactive peptide content (J. Cao et al., 2022).

Industrial chemical contamination has harmed mammalian reproductive health worldwide. These toxins' ubiquitous environmental presence endangers numerous creatures' reproductive systems (Di Renzo et al., 2015). Fertility, reproductive function, and health are concerns for researchers and doctors. Understanding how industrial pollutants damage reproductive systems is vital to establishing effective mitigation techniques and maintaining mammalian reproductive health (Zhang et al., 2016). Lead exposure affects the liver, kidneys, reproductive system, haematological system, central and peripheral neurological system, cardiovascular system, endocrine glands, and behavioural and biochemical functioning of humans and animals

(Albishtue et al., 2020). Several investigations identified toxicological effects on haematopoiesis, central nervous system, kidneys, liver, reproductive system, and cardiovascular system (Singh et al., 2013, Renieri et al., 2019). Pb-exposed mice had more atretic and less primordial follicles in their ovaries (Sharma et al., 2012). Prolonged exposure to PbA can lead to endocrine dysfunction characterized by reduced levels of LH and FSH (Hamadouche et al., 2013; Assi et al., 2016; Assi et al., 2017). Pb causes severe harm to the hypothalamic-pituitary axis because it suppresses growth hormone (GH), which lowers somatotrope receptivity (Ahmed et al., 2013, Shojaepour et al., 2018, Singh et al., 2018). Lead, cadmium and arsenic are environmental pollutants that may contribute to hormonal disruption, cause reproductive disorders and risk factors for infertility in women (Hsiao et al., 2015)

PbA is a hazardous chemical that accumulates in animal tissues and has a lengthy half-life. Lead is 90% in bones and 4% in blood. Lead toxicity is largely induced by the liver and kidneys' lead elimination (Deavall et al., 2014; Renieri et al., 2019). Mammalian exposure is usually assessed through the measurement of lead in blood (Samuel et al., 2022). Preterm birth, low birth weight, infertility, miscarriage, neurological problems in infants, and maternal hypertension are hazards for women with a 10 µg/dL BPb. Lead-exposed rats also showed reduced reproductive organ activity of the fertility-decreasing enzymes sodium potassium ATPase and alkaline phosphatase (ALP). The study reported by Shafiq et al. (1984) that Pb concentration increases lipid peroxidation and liver tissues (Shafiq et al., 1984; Benoff et al., 2000). Over 90% of Pb body burden resides in the skeleton at constant state. The liver and kidney receive the most Pb from blood plasma. Dense cortical bone contains Pb because the PNS accumulates more than the CNS. Second, soft tissues and

mitochondrial-active organs have greater Pb contents. The renal tubule, CNS choroid plexus, and cerebellum have the most mitochondrial activity.

Chinese people have employed swiftlet saliva-derived EBN to heal for millennia. Edible bird's nest is a nutrient-rich food supplement in Asia that boosts energy, slows aging, and improves health. Albishtue et al. (2019) found that EBN improved reproductive performance, pregnancy success, and lead acetate-induced reproductive harm (Albishtue et al., 2019). Studying how PbA exposure affects reproductive dysfunction and using natural fecundity boosters is vital. EBN improves mammalian reproduction and fertility by counteracting heavy metals (Kong et al., 1989). EBN's antioxidant and regeneration properties have been widely studied in this research. EBN hydrolysates are made enzymatically. EBN's poorly water-soluble proteins, glycoproteins, and polysaccharides can be converted into less complicated and more easily absorbed free peptides, glycopeptides, and free sugar chains (e.g., mannose, galactose, N-acetylgalactosamine, and N-acetylglucosamine) (Fan et al., 2022; Yan et al., 2022). "Edible bird's nest peptides" are the peptides. Hydrolysis can also improve bioavailability of bioactive compounds like conjugated sialic acid and N-acetylneuraminic acid in EBN (Wong et al., 2018).

EBN's antioxidant, anti-inflammatory, and immunomodulatory activities have drawn scientific attention. Hydrolysis releases bioactive peptides from EBN, potentially improving bioavailability and health effects. There is little study on how raw and hydrolyzed EBN mitigate lead acetate-induced reproductive damage. Thus, this study compared raw and hydrolyzed EBN in protecting rats' reproductive systems against lead acetate. This study examined hormonal profiles and reproductive organ histology to see whether EBN may mitigate lead acetate's negative effects on reproductive health.

6.2 Materials and methods

6.2.1 EBN and Lead acetate preparation

The preparation of R and H EBN and lead acetate was carried out in the same manner as stated in Chapter 3.

6.2.2 Experimental design

For this study, we housed eight groups of six adult female SD rats (12 weeks old) in cages. After acclimating the rats for seven days, they were randomly split into eight groups of six animals each and exposed to lead acetate (PbA) for eight weeks while also receiving an in vivo supplement of R & H EBN according to Table 6.1 below. According to Albishtue et al. (2019) research, the doses of R and H EBN were selected based on research published by Histological tests were performed to count the expressions of LE, GE and S in uterine and OSE, CL and follicles in ovarian tissues, liver and kidney tissues, and blood cells, as described in Chapter 3.

Table 6.1: Animal grouping and treatment

Group	Group assigned	Type of treatment (dose)/8 weeks
Control Treatment Group	G1 (Control)	Normal diet +Distill water
	G2 (Positive control)	PbA only (10mg/kg)
	G3 (Low dose)	PbA + REBN (10mg/kg + 60mg/kg bwt.)
	G4 (Medium dose)	PbA + REBN (10mg/kg + 90mg/kg bwt.)
	G5(High dose)	PbA + REBN (10mg/kg + 120mg/kg bwt.)
	G6 (Low dose)	PbA + HEBN (10mg/kg + 20mg/kg bwt.)
	G7 (Medium dose)	PbA + HEBN (10mg/kg + 40mg/kg bwt.)
	G8 (High dose)	PbA + HEBN (10mg/kg + 60mg/kg bwt.)

ND=Normal diet, PbA=Lead Acetate, REBN= Raw edible bird’s nest, HEBN= Hydrolysed edible bird’s nest, bwt=Body weight.

6.2.3 Monitoring of oestrous cycle phases and the oestrous synchronization

The oestrous cycle phases, and the estrous synchronization determination were carried out in the manner outlined in Chapter 3.

6.2.4 Macro and microscopic inspections of the uterus, ovaries, kidney, and liver

Macro and microscopic inspections of the uterus, ovaries, kidney, and liver were accomplished as described in Chapter 3.

6.2.5 Effect of REBN and HEBN of E2 and P4 hormones

Analyses of the concentrations of plasma for E2 and P4 were completed as described in Chapter 3.

6.2.6 Statistical analysis

Graph Pad Prism 8.0 calculated the mean (M) and standard error of the mean (SEM). Graph Pad Software, San Diego. The ratio of ovarian to uterine bwt, SOD levels, and PbA levels in uterine tissues were assessed using a one-way ANOVA and a Tukey multiple comparison post-hoc test. bwt used two-way ANOVA and Bonferroni's multiple comparison testing. P values less than or equal to 0.05 indicated statistical significance.

6.3 Results

6.3.1 Effect of REBN and H EBN on body weight gain (BWG)

The bwts of the rats in each group during the experiment were noticeably different from one another, despite the fact that PbA-exposed animals exhibited a regular (4-5-day) oestrous cycle and no changes in the cell morphologies of the vaginal cytology. In week 8, the REBN of 120 mg/kg bwt (G5) and HEBN of 60 mg/kg bwt (G8) groups varied substantially ($p < 0.05$) from the untreated control group (G1) and other EBN treated groups, with the positive control (G2) having a lower bwt and the REBN groups having a greater bwt (Figure 6.1).

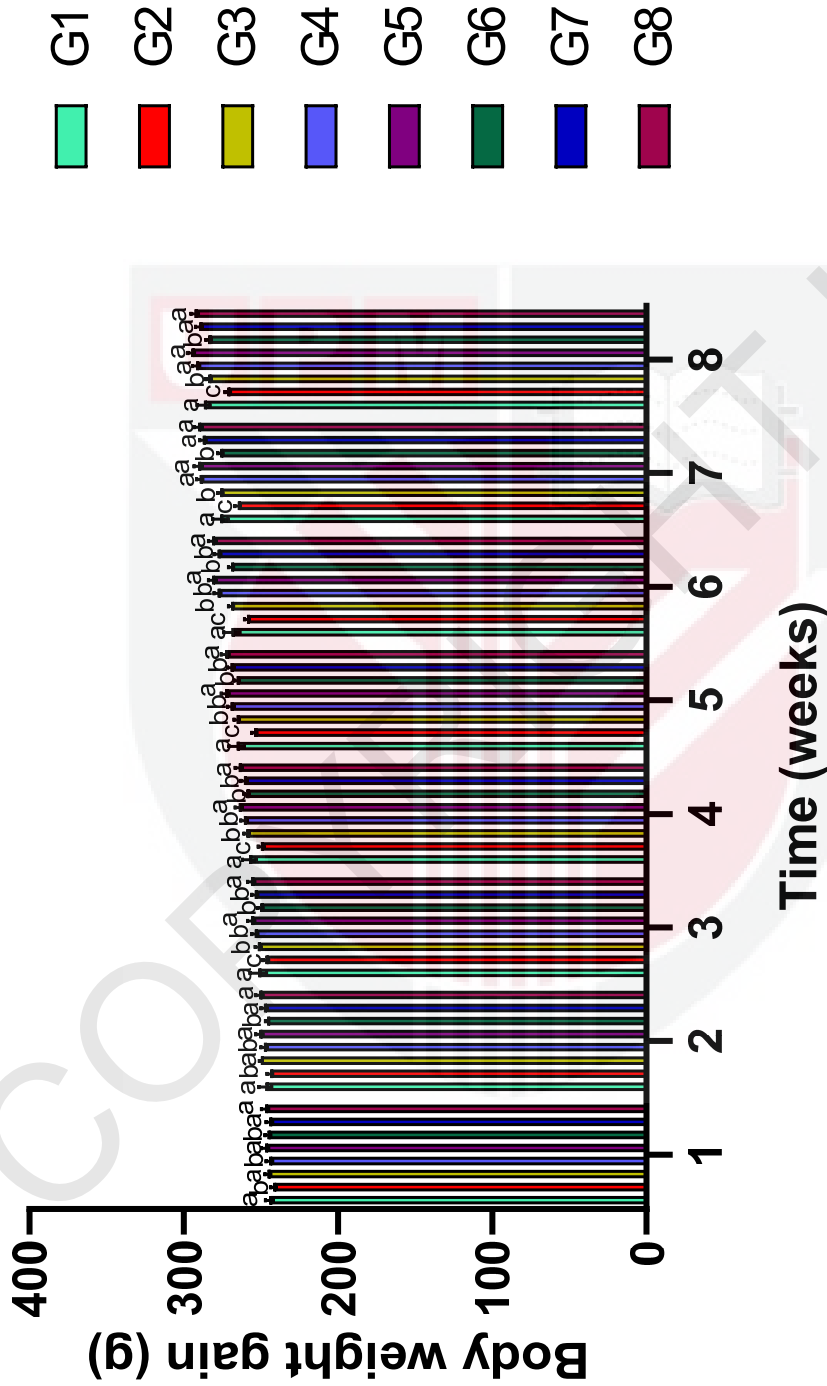


Figure 6.1: Effect of Raw and Hydrolyzed EBN on body weight gain (g) of rats of R and H EBN treated groups compared to control and PbA group

Data is expressed as Mean $\pm$ SEM. Error bars showing different letters a, b and c within rows for each week denote a significant difference at $p < 0.05$. *G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 90 mg/kg bwt) G4= (REBN 120 mg/kg bwt), G5= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt).

6.3.2 The effect of the REBN and HEBN on the uterine and ovarian bwt ratio (UBWR) & (OBWR)

Compared to the EBN treatment groups and the control (G1), the positive control (G2) had a lower UBWR, whereas the REBN of 120 mg/kg bwt (G5) group had a significantly ($p < 0.05$) higher UBWR, in comparison with treatment groups and control (G1). For treatment groups, the alterations rose considerably ($p < 0.05$) and reached the greatest value in the REBN of 120 mg/kg bwt (G5) & HEBN of 60 mg/kg bwt (G8) groups (Figure 6.2A). When the ovarian body weight ratios (OBWR) of the R and HEBN dose groups were correlated with control and the PbA groups, G2 (positive control) group had a lower OBWR and the REBN 120 mg/kg bwt (G5) group with higher OBWR ($p < 0.05$). For treated groups, changes significantly rose and reached greatest value ($p < 0.05$) in REBN of 120 mg/kg bwt (G5) group (Figure 6.2B).

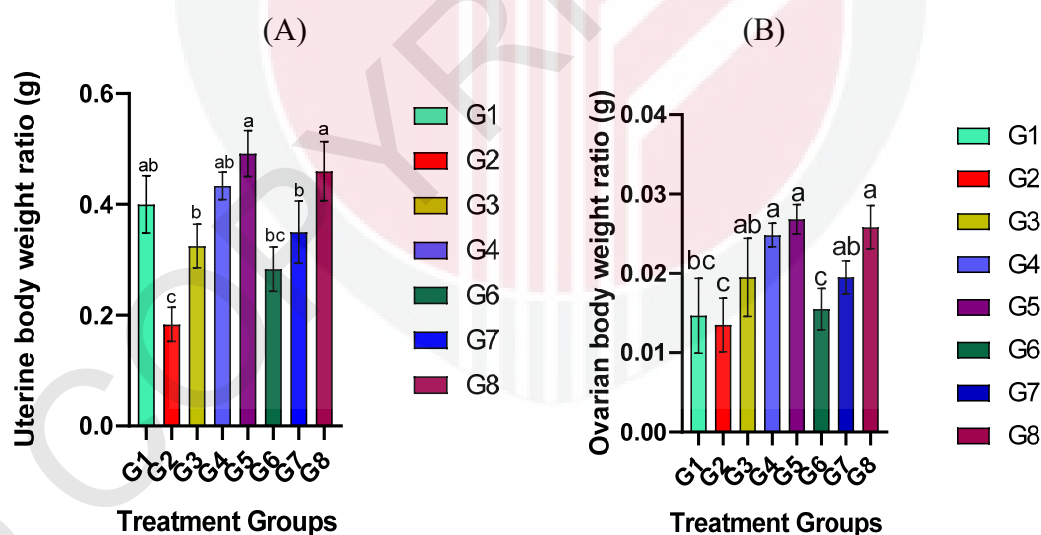


Figure 6.2: Effect of PbA and Raw and Hydrolyzed EBN treated groups on Uterine body weight ratio (A) and Ovarian body weight ratio (B) when compared to control and PbA group

Data are expressed as Mean $\pm$ SEM. Error bars showing different letters a, b and c within rows denote a significant difference at $p < 0.05$. *G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt).

EBN increased the uterus' weight and length dose-dependently. In comparison to control, the PbA-treated (G2) group had a decline in luminal epithelial thickness. However, the REBN of 120 mg/kg bwt (G5) and the HEBN of 60 mg/kg bwt (G8) groups presented considerably ($p < 0.05$) larger LE thicknesses in relation to all other treated groups ($p < 0.05$) (Figure 6.3).

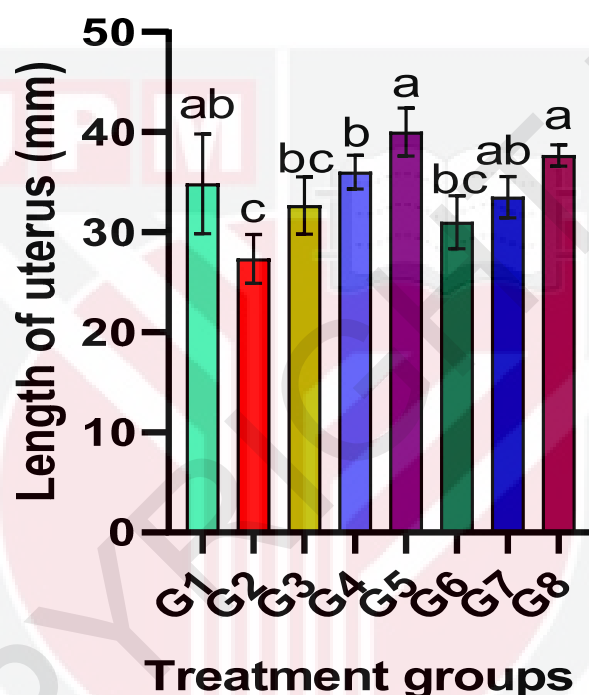


Figure 6.3: Effect of PbA on Uterine body length of Raw and Hydrolyzed EBN treated groups when compared to control and PbA group

Data are expressed as Mean $\pm$ SEM. Error bars showing different letters a, b and c within rows denotes a significant difference at $p < 0.05$. *G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt).

6.3.3 Thickness of luminal epithelium

Thickness of the LE in group of G5 (120 mg/kg bwt) treated with REBN increased.

Dose-dependently, EBN increased the thickness of LE. Considerable difference ($p <$

0.05) was observed of the LE thickness for REBN of 120 mg/kg bwt (G5) and HEBN of 60 mg/kg bwt (G8) when compared to all EBN treatment and negative control groups (Figure 6.4). The result of the present study found that G2 (positive control) had the lowest value of any group, both the control and treatment groups (Figure 6.4).

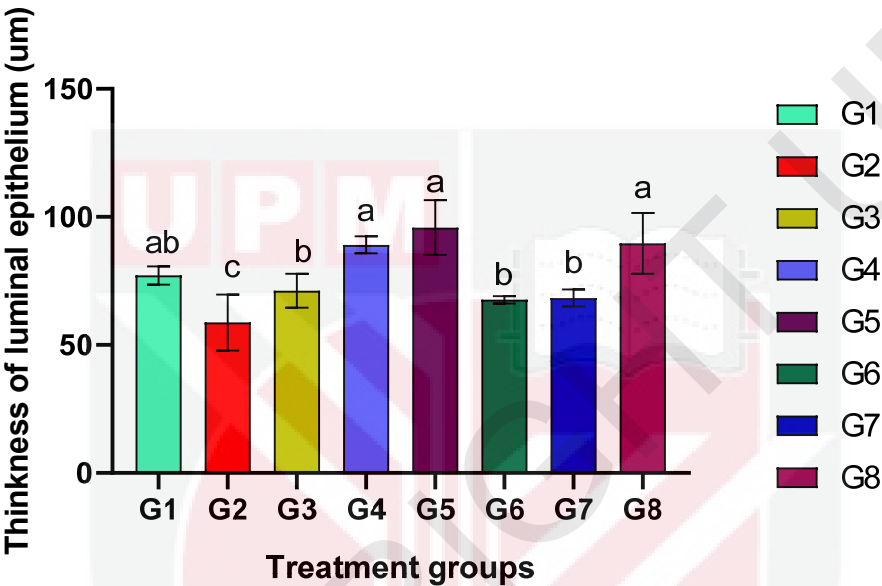


Figure 6.4: Effect of PbA on Raw and Hydrolyzed EBN on thickness of luminal epithelium (LE) on treated groups when compared to control and PbA group
 Data are expressed as Mean±SEM. Error bars showing different letters a, b and c within rows denotes a significant difference at p<0.05. *G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt).

Increased numbers for embryo implantation sites were observed in G4, G5, for dosage values 90 and 120 mg/kg bwt of REBN, whereas in G8 at dosage values 60 mg/kg of HEBN, in comparison to control and PbA-only groups (Figure 6.5).

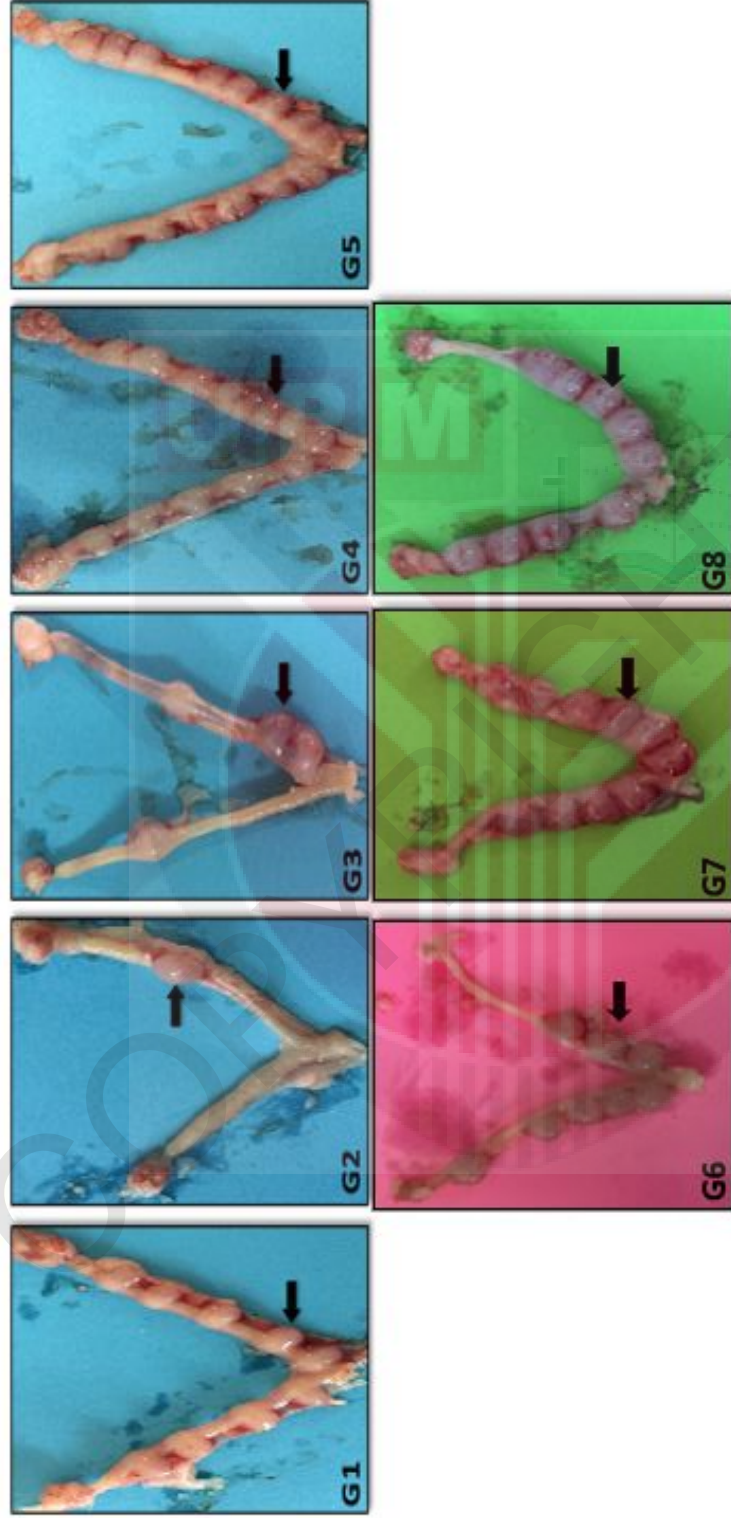


Figure 6.5: Representative picture of gravid uterus from all experimental groups showing embryo implantation sites. Black arrow indicates implantation sites

*G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt).

6.3.4 Embryo implantation rate and Fertility index

Embryo implantation sites (EIR) and fertility index (%) were measured on day eight of pregnancy in rats and compared to a control group. A statistically significant difference was found between the REBN 90 mg/kg bwt (G4) and REBN 120 mg/kg bwt (G5) groups in terms of the fertility index and the number of EIR, but not between the control (G1), positive control (G2), REBN 60 mg/kg (G3), HEBN 20 mg/kg bwt (G6), and HEBN 40 mg/kg bwt (G7) groups (Figure 6.7). The highest fertility index values ($p<0.05$) were found in the groups exposed to 120 mg/kg bwt of REBN (Group 5) and 60 mg/kg bwt of HEBN (Group 8), as shown in (Figure 6.6).

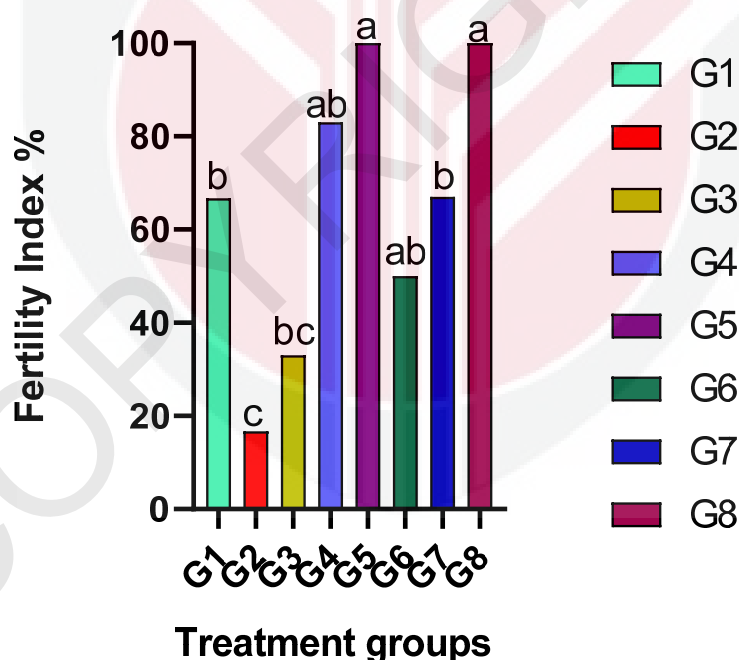


Figure 6.6: Impact of Raw and Hydrolyzed EBN on the Fertility index Percentage (%) of R and HEBN treated groups when compared to control and PbA group

Bar graphs showing different letters a, b and c within rows denotes a significant difference at $p<0.05$. *G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) G4= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). *Fertility index was calculated according to (Albishtue et al., 2018) number of pregnant females/number of females with confirmed mating x 100.

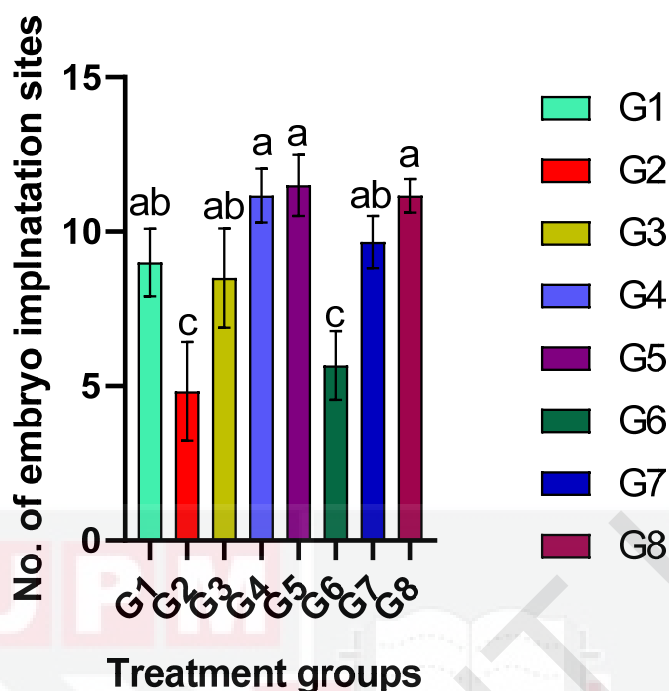


Figure 6.7: No. of implantation sites of the Raw and Hydrolzed EBN treated groups when compared to the control and PbA group

Data are expressed as Mean±SEM. Error bars showing different letters a, b and c within rows denotes a significant difference at $p < 0.05$. *G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt).

6.3.5 Histopathological examination of uterine and ovarian tissues

The uterus was found to have no obvious gross abnormal lesions. In epithelial cells degeneration and the apoptosis was observed, and number of the endometrial glands declined in PbA treated positive control group and the also in lowest R and HEBN treated groups, but histopathological examination of uteri from other groups (except G5 and G8) showed Pb-induced inflammatory changes that were characterised by endometrium atrophy, vacuolar degeneration, inflammatory changes in (G2) PbA treated group. There were two exceptions to this: REBN of 120 mg/kg bwt (G5) & HEBN of 60 mg/kg bwt (G8) groups revealed normal uterine anatomy. As shown in Figure 6.9 shows improved uterine endometrial structures at a dose of REBN of 120

mg/kg bwt (G5) and HEBN of 60 mg/kg bwt (G8) groups than in positive control (G2) and the other groups observed. Histopathology slides showed cuboidal or flattened cells in the uterine lining and depicted an increased number of uterine glands when treated at a concentration of 120 mg/kg bwt of the REBN (G5) and 60 mg/kg bwt dose of HEBN (G8). Furthermore, mean score differences of histopathological lesions in the uterus among the groups are shown in (Figure: 6.8) and (Table 6.2).



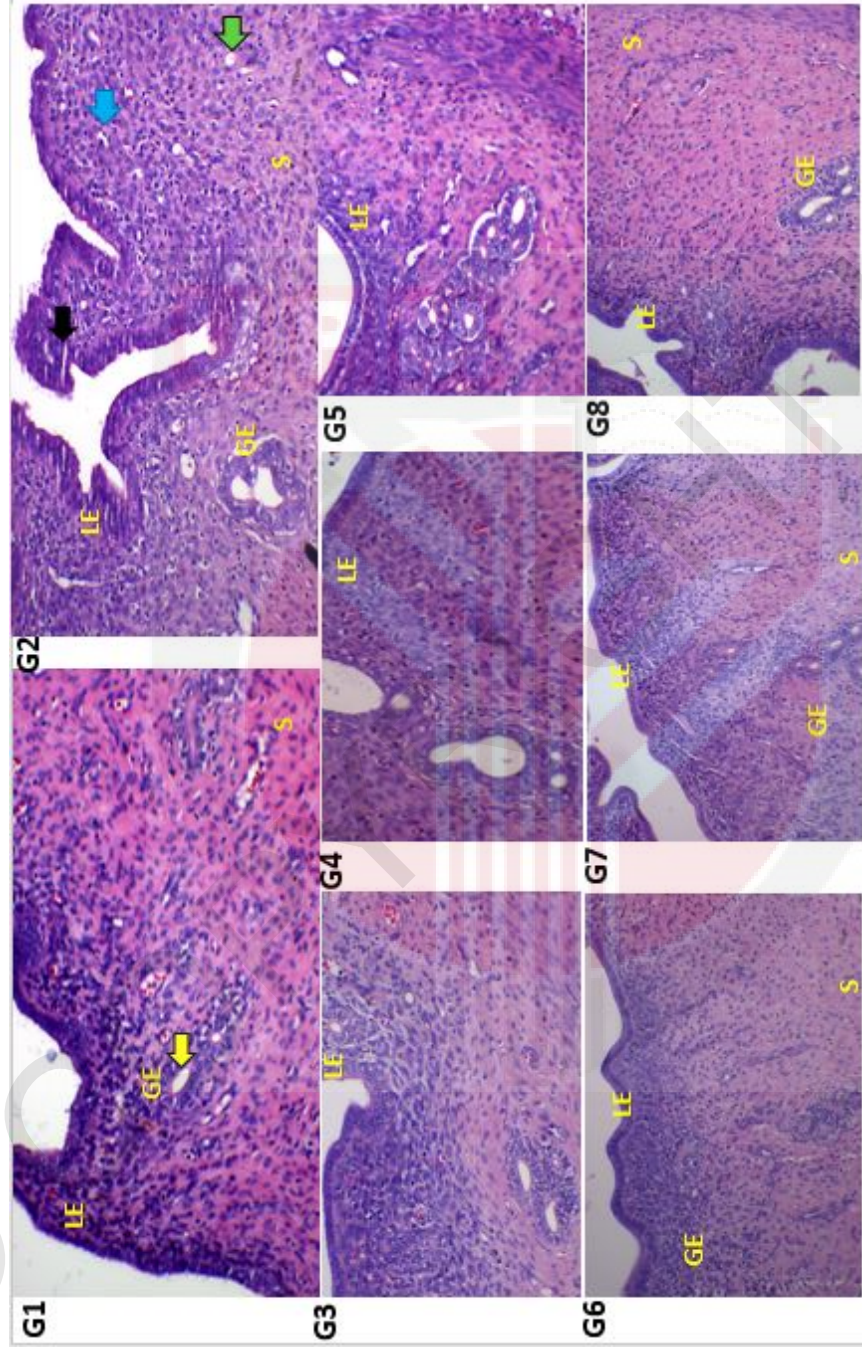


Figure 6.8: PbA exposure and effect of Raw and Hydrolyzed EBN on uterine histomorphology

*G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) and G4= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). Note, (LE) signify luminal epithelium, (S) stroma and (GE) Glandular Epithelium. Yellow arrows indicate normal uterine glands while blue arrows indicate vasoconstriction, and green arrow indicates degeneration of LE cells (Black arrow) is evident in G2 compared to R and HEBN treated groups. H&E stain. Magnification 200x.

Table 6.2: Effect of different levels of raw and hydrolyzed edible bird’s nest fed to lead acetate (PbA)-exposed rats on the score of pathological changes in the uterine tissue (Mean ± SEM)

Parameters	Groups							
	G1	G2	G3	G4	G5	G6	G7	G8
Vacuolation	0±0 ^a	2.5±0.5 ^c	1.6±0.3 ^b	0±0 ^a	0±0 ^a	2±0 ^b	1.1±0.4 ^a	0±0 ^a
Necrosis of LE and GE	0±0 ^a	2.6±0.3 ^c	1.8±0.1 ^b	0.6±0.1 ^a	0±0 ^a	2.1±0.1 ^c	0.6±0.1 ^a	0±0 ^a
Infiltration of inflammatory cells	0±0 ^a	2.8±0.1 ^c	2.3±0.1 ^c	1.3±0.3 ^b	0.1±0.1 ^a	1.8±0.1 ^b	1.3±0.3 ^b	0.6±0.3 ^a
Congestion	0±0 ^a	3±0 ^c	2.1±0.1 ^c	0±0 ^a	0±0 ^a	2±0 ^b	1±0 ^a	0.3±0.3 ^a

Means with different superscripts within each row differ significantly (P<0.05).

*G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) G4= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt)

However, the histopathological inspection of the ovary showed fat lobules in G2 (positive control) and REBN of 60 mg/kg bwt (G3) groups. Similarly, antral degeneration and congestion were shown in G2 (positive control) and REBN of 60 mg/kg bwt (G3) groups compared to treatment groups (Figure 6.9). Furthermore, the mean score differences of histopathological lesions in the ovary among the groups are shown in Table 6.3.

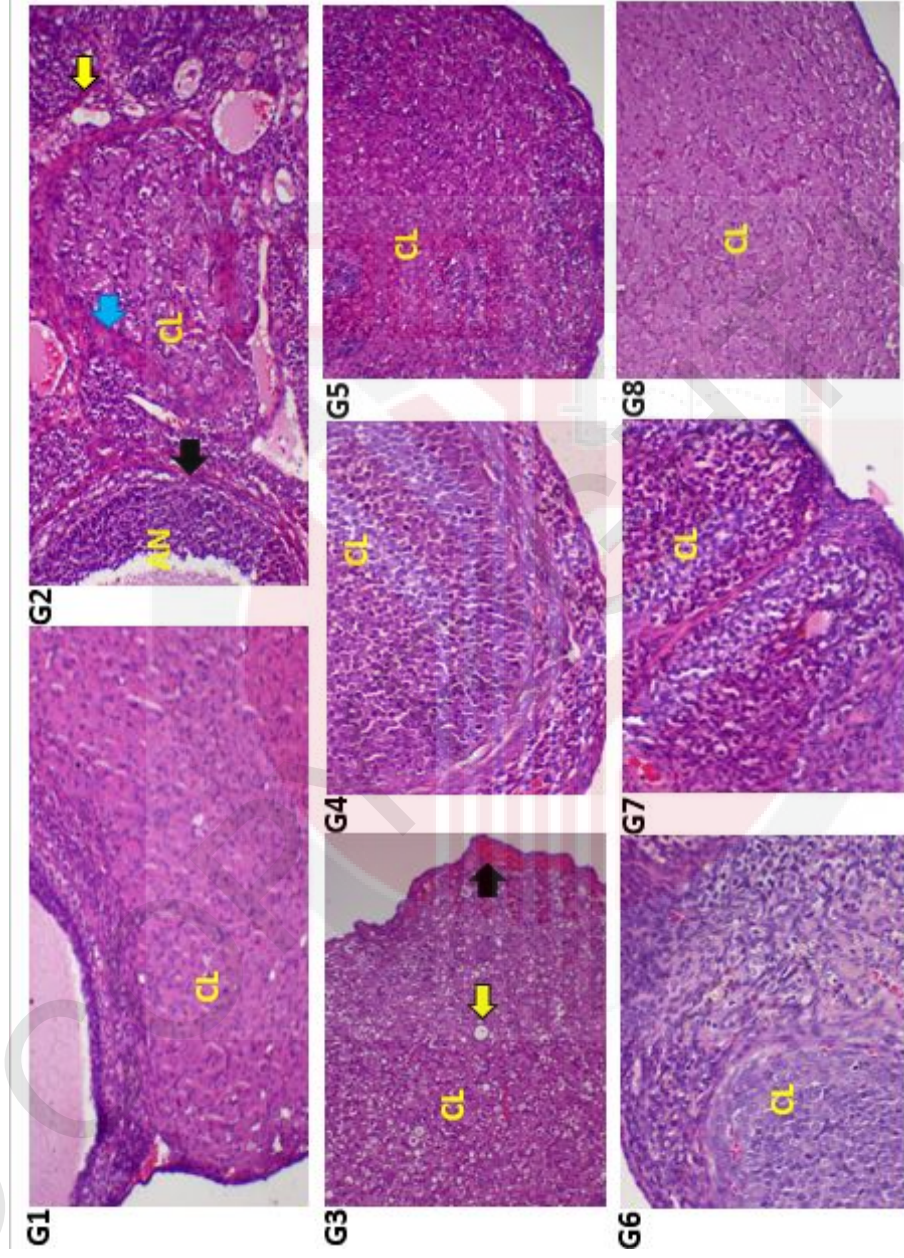


Figure 6.9: PbA exposure and effect of Raw and Hydrolyzed EBN on ovarian histomorphology

*G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt); G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). Note, (CL) signify corpus luteum, (AN) antral follicle. Yellow arrows indicate fat lobules while blue arrows indicate congestion. Degeneration of antral follicle in black arrow is evident in G2 compared to R and HEBN treated groups. H&E stain. Magnification 200x.

Table 6.3: Effect of different levels of raw and hydrolyzed edible bird's nest fed to lead acetate (PbA)-exposed rats on the score of pathological changes in the ovarian tissue (Mean ± SEM)

Parameters	Groups							
	G1	G2	G3	G4	G5	G6	G7	G8
Vacuolation	0±0 ^a	2.6±0.3 ^c	2.1±0.6 ^b	0±0 ^a	0±0 ^a	2±0 ^b	1.6±0.2 ^b	0±0 ^a
Inflammation of Follicle Atresia	0±0 ^a	2.8±0.1 ^c	2.3±0.3 ^b	0.1±0.1 ^a	0±0 ^a	2±0 ^b	1±0 ^a	0±0 ^a
Infiltration of inflammatory cells	0±0 ^a	2.5±0.2 ^c	2±0 ^b	0.5±0.2 ^a	0.1±0.1 ^a	1.6±0.2 ^b	2±0 ^b	0.5±0 ^a
Congestion	0±0 ^a	3±0 ^c	2.6±0.3 ^c	0.6±0.3 ^a	0±0 ^a	2.6±0.2 ^c	1.6±0.1 ^b	0±0 ^a

Means with different superscripts within each row differ significantly (P<0.05).
*G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) G4= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt).

6.3.6 Histopathological examination of kidney and liver tissues

When negative and positive control groups were compared to the R and H EBN treated groups, light microscopy analysis of kidney samples from G1 (control) revealed a normal histological structure of the renal parenchyma (Figure 6.11). The mean scores of histopathological lesions in the kidney show a significant amount of necrosis in the G2 (positive control), G3 (REBN 60 mg/kg bwt), G6 (HEBN 20 mg/kg bwt), and G7 (HEBN 40 mg/kg bwt) groups (Table 6.4). However, G2 (positive control), G3 (REBN 60 mg/kg bwt), G6 (HEBN 20 mg/kg bwt) and G7 (HEBN 40 mg/kg bwt) showed significant degeneration of bowmen's capsule of kidney tissues when compared to negative control and highest R and HEBN treated G4 and G5 (REBN of 90 and 120 mg/kg bwt) and G8 (HEBN of 60 mg/kg bwt) groups. As shown in (Figure 6.10).

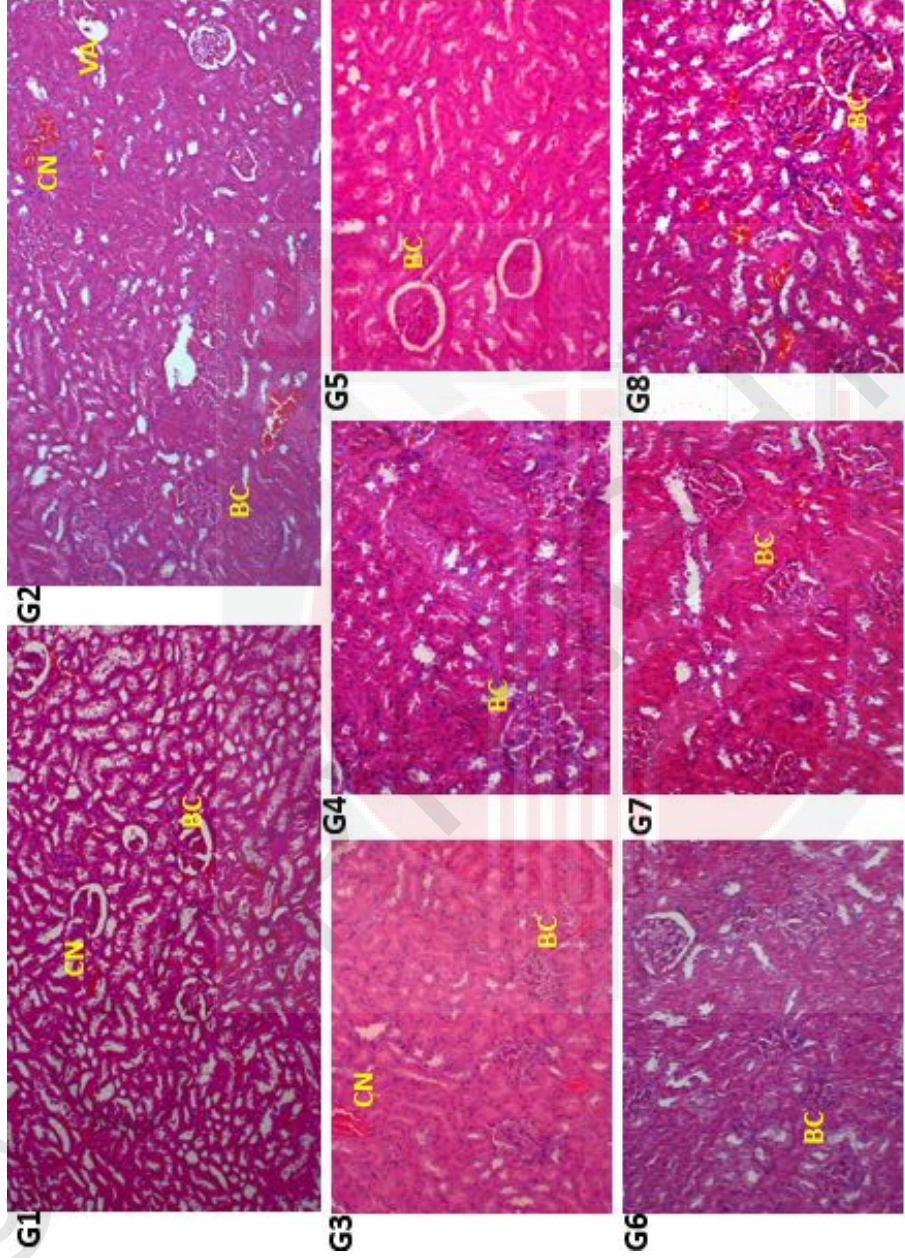


Figure 6.10: PbA exposure and effect of Raw and Hydrolyzed EBN on kidney histomorphology

*G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt); G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). (CN) signify necrosis, (VA) signify as vacuolation, (BC) as bowmen's capsule is evident in G2, G3, G6, G7 when compared to negative control and R and HEBN treated groups. H&E stain. Magnification 200x.

Table 6.4: Effect of different levels of raw and hydrolyzed edible bird's nest fed to lead acetate (PbA)-exposed rats on the score of pathological changes in the kidney tissue (Mean $\pm$ SEM)

Parameters	Groups							
	G1	G2	G3	G4	G5	G6	G7	G8
Vacuolation	0 $\pm$ 0 ^a	2.3 $\pm$ 0.3 ^c	2 $\pm$ 0 ^b	0.1 $\pm$ 0.1 ^a	0 $\pm$ 0 ^a	1.1 $\pm$ 0.1 ^b	1 $\pm$ 0 ^a	0 $\pm$ 0 ^a
Necrosis	0 $\pm$ 0 ^a	2.5 $\pm$ 0.2 ^c	1.8 $\pm$ 0.1 ^b	0 $\pm$ 0 ^a	0 $\pm$ 0 ^a	1.1 $\pm$ 0.1 ^b	1 $\pm$ 0 ^a	0 $\pm$ 0 ^a
Infiltration of inflammatory cells	0 $\pm$ 0 ^a	2.1 $\pm$ 0.2 ^c	2.3 $\pm$ 0.1 ^c	0.6 $\pm$ 0.3 ^a	0.1 $\pm$ 0.1 ^a	2 $\pm$ 0.5 ^b	1.6 $\pm$ 0.3 ^b	0.3 $\pm$ 0.3 ^a
Degenerative bowman's capsule	0 $\pm$ 0 ^a	3 $\pm$ 0 ^c	2.8 $\pm$ 0.1 ^c	0 $\pm$ 0 ^a	0 $\pm$ 0 ^a	2.8 $\pm$ 0.1 ^c	0.3 $\pm$ 0.3 ^a	0 $\pm$ 0 ^a
Congestion	0 $\pm$ 0 ^a	2.6 $\pm$ 0.3 ^c	1.6 $\pm$ 0.3 ^b	0.6 $\pm$ 0.3 ^a	0 $\pm$ 0 ^a	2 $\pm$ 0 ^b	1 $\pm$ 0.5 ^a	0 $\pm$ 0 ^a

Means with different superscripts within each row differ significantly (P<0.05).

*G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) G4= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt)

Following the light microscopic examination of liver tissues from the various experimental groups, a typical structural organization, abundant binucleated hepatocytes, absence of inflammatory cell infiltration, and normal central veins were found in the control group (G1) (Figure 6.11). Mean scores of histopathological lesions in the liver showed that there was a significant activation of the kupffer cells in the G2 (positive control), G3 (REBN 60 mg/kg bwt), G6 (HEBN 20 mg/kg bwt), and G7 (HEBN 40 mg/kg bwt) groups (Table 6.5). Whereas G2 (positive control) G3 (REBN 60 mg/kg bwt) and G6 (HEBN 20 mg/kg bwt) showed significant changes in liver tissue with necrosis (Figure 6.11).

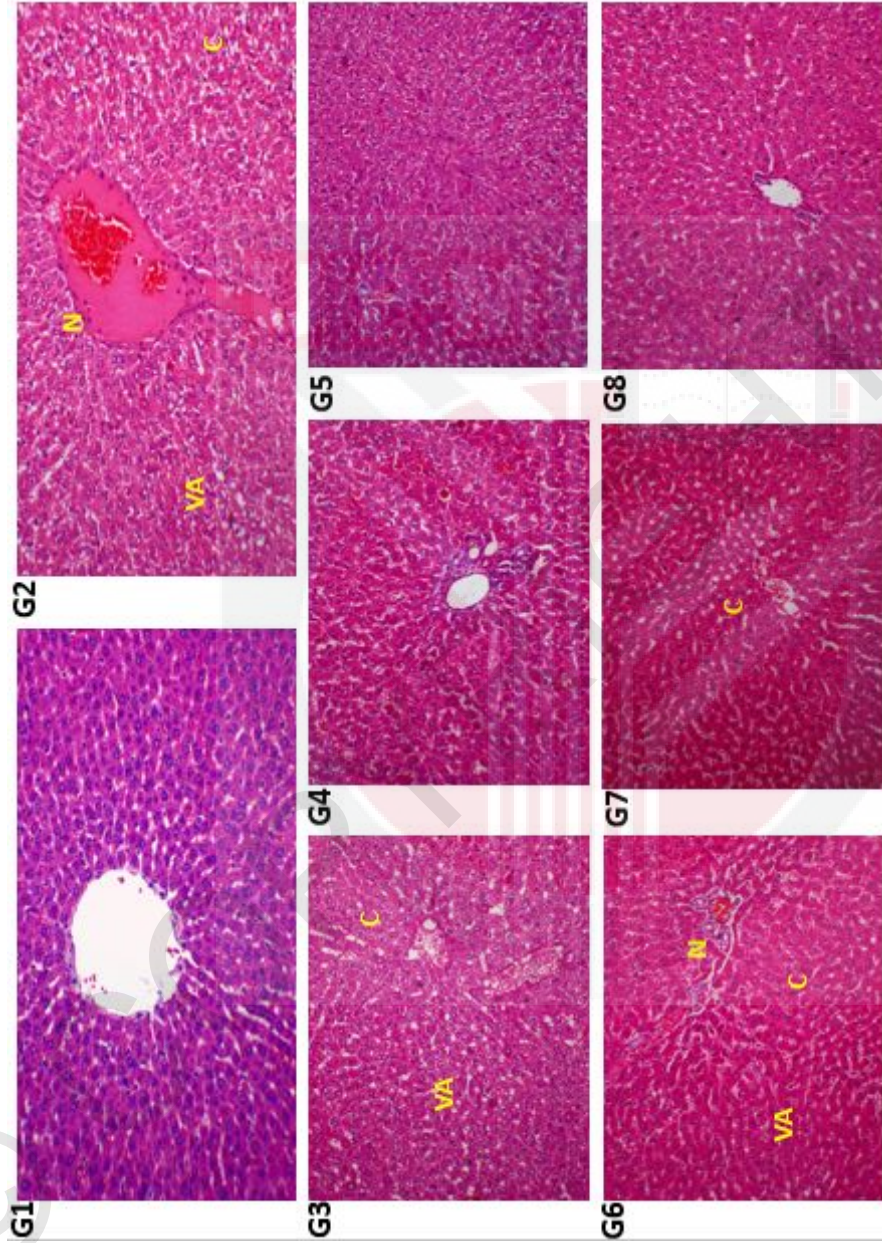


Figure 6.11: PbA exposure and effect of Raw and Hydrolyzed EBN on liver histomorphology

*G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). (N) signify necrosis, (VA) as vacuolation and (C) as congestion is evident in G2, G3, G6, G7 when compared to negative control and R and HEBN treated group. H&E stain. Magnification 200x.

Table 6.5: Effect of different levels of raw and hydrolyzed edible bird's nest fed to lead acetate (PbA)-exposed rats on the score of pathological changes in the liver tissue (Mean $\pm$ SEM)

Parameters	Groups							
	G1	G2	G3	G4	G5	G6	G7	G8
Vacuolation	0 $\pm$ 0 ^a	2.8 $\pm$ 0.1 ^c	2.3 $\pm$ 0.1	1 $\pm$ 0 ^a	0 $\pm$ 0 ^a	2 $\pm$ 0 ^b	1 $\pm$ 0 ^a	0 $\pm$ 0 ^a
Necrosis	0 $\pm$ 0 ^a	2.6 $\pm$ 0.3 ^c	1.8 $\pm$ 0.1	0 $\pm$ 0 ^a	0 $\pm$ 0 ^a	1.6 $\pm$ 0.3 ^b	0.6 $\pm$ 0.3 ^a	0 $\pm$ 0.1 ^a
Infiltration of inflammatory cells	0 $\pm$ 0 ^a	2.8 $\pm$ 0.1 ^c	1.1 $\pm$ 0.4	0.5 $\pm$ 0.2 ^a	0.3 $\pm$ 0.1 ^a	2.3 $\pm$ 0.3 ^c	1 $\pm$ 0 ^a	0.3 $\pm$ 0.5 ^a
Activation of Kupffer cells	0 $\pm$ 0 ^a	2.6 $\pm$ 0.3 ^c	0.6 $\pm$ 0.1	0.3 $\pm$ 0.3 ^a	0 $\pm$ 0 ^a	2 $\pm$ 0 ^b	0.3 $\pm$ 0.3 ^a	0 $\pm$ 0 ^a
Congestion	0 $\pm$ 0 ^a	3 $\pm$ 0 ^c	1.3 $\pm$ 0.3	0 $\pm$ 0 ^a	0 $\pm$ 0 ^a	1.6 $\pm$ 0.3 ^b	1 $\pm$ 0 ^a	0.3 $\pm$ 0.3 ^a

Means with different superscripts within each row differ significantly ($P < 0.05$).

*G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) G4= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt)

6.3.7 Concentrations of Plasma for E2 and P4

Oestrogen was observed significantly ($p < 0.05$) lower in the positive control group and higher ($p < 0.05$) in highest R and the HEBN dose groups (REBN of 120 mg/kg bwt) and (HEBN 60 mg/kg bwt) when compared to other R and H EBN treated groups, negative and positive controls. For treatment groups, the variations considerably increased and reached greatest value ($p < 0.05$) in G5 (Figure 6.12). Similarly, the P4 was lesser in positive control group (G2) and greater ($p < 0.05$) in G5 (REBN of 120 mg/kg bwt) and G8 (HEBN 60 mg/kg bwt) groups when R and H EBN treated groups, negative and positive control groups. (Figure 6.12).

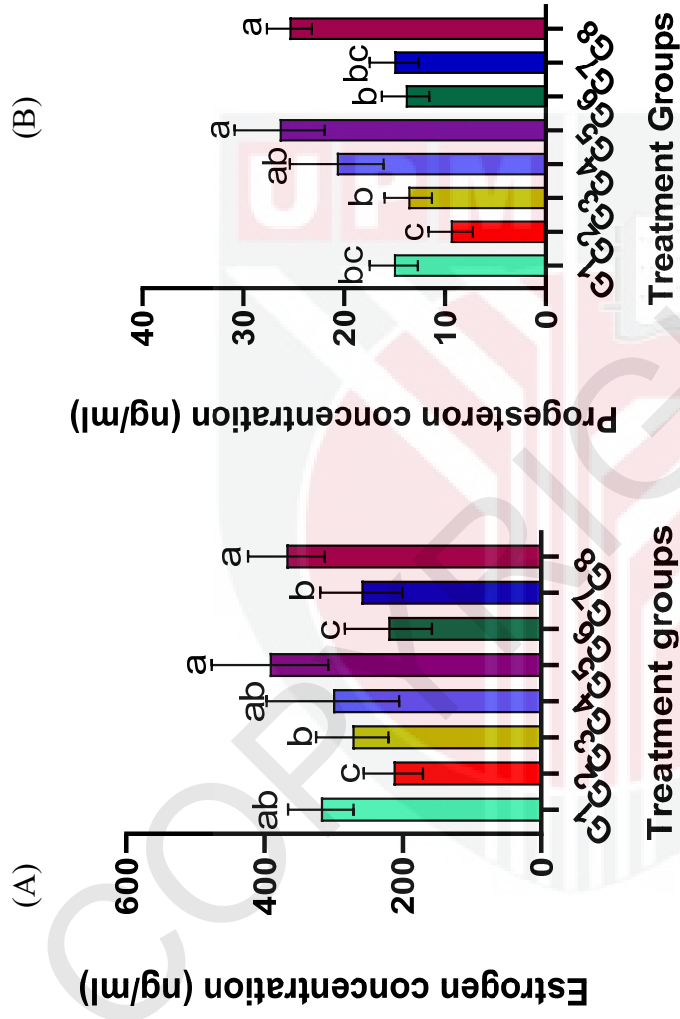


Figure 6.12: Effect of the Raw and Hydrolyzed EBN on concentrations of E2 and P4 in rats exposed to PbA toxicity

This data shows plasma concentrations of estrogen hormone (A), progesterone hormone (B), in rats supplemented with different doses of EBN. Data are expressed as Mean $\pm$ SEM. Error bars showing different letters a, b and c within rows denote a significant difference at $p < 0.05$. *G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) G4= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt), G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt).

6.4 Discussion

In this study, we compared the preventive effects of EBN in its raw and hydrolysed forms against lead acetate-induced reproductive harm in rats. This study was aimed to clarify the possible benefits of EBN in reducing the negative effects of lead acetate on the reproductive system by the evaluation of numerous reproductive indicators, including hormonal profiles, and histological changes in reproductive organs. To the best of our knowledge, this study represents the first investigation into the impact of short-term PbA poisoning on the reproductive function of rats and the potential mitigating effects of EBN supplementation.

A well-known poisonous element called lead acetate has been linked to harmful effects on both human and animal reproduction. Pb's toxic effects are especially dangerous to the reproductive system because it can alter hormone synthesis, impede sperm function, and alter the histopathology of reproductive organs. Therefore, it is crucial to find efficient preventative methods against lead's effects on reproductive toxicity. Due to their rich protein, amino acid, mineral, and bioactive peptide compositions, raw and hydrolysed EBN were chosen as potential protective agents in this study. In the past, people have consumed raw EBN for its alleged health advantages, however hydrolysed EBN goes through a procedure that increases its bioavailability and produces bioactive peptides that could have physiological effects.

Lead is absorbed into the blood and attaches to erythrocytes for most of its time before diffusing into bones and other soft tissues, where it mixes with blood lead and accumulates to lethal levels (Ali et al., 2020). The vaginal cytological investigation

demonstrated a normal oestrous cycle regardless of the subjects' exposure to PbA along with EBN treatment in present study. A recent study highlighted that the EBN supplementation had had a positive impact on the uterus of normal rats (Albishtue et al., 2019). PbA exposure impairs menstruation, reduces fertility, delays conception, alters hormonal production, blood circulation, and impacts pregnancy and its result. Pre-implantation embryo loss may result from chemical harmful effects. Lead acetate has been shown to decrease implantation sites and increase reabsorptions (Bataineh et al., 2007; Kumar et al., 2018).

Toxicological research requires body and organ weight analysis (Höfer et al., 2009). The findings of this study revealed that the administration of PbA at a dosage of 10 mg/kg bwt for a duration of eight weeks resulted in detrimental effects on bwt, uterine body weight ratio (UBWR), and ovarian body weight ratio (OBWR). Previous research had also indicated that exposure to low to moderate levels of PbA adversely affected the bwt of female rats (Uchewa et al., 2019; Massányi et al., 2020). A study conducted by Abubakar et al. (2019) discovered a connection between bodyweight loss and PbA levels in the pituitary and hypothalamus of PbA-exposed mice (Abubakar et al., 2019). It is possible that the generation of free radicals, which causes oxidative stress, is related to the decrease of bwt brought on by PbA exposure (Lee et al., 2019). All EBN-treated rats were protected from PbA-induced wasting and bodyweight loss, as seen by noticeably larger bwt gains compared to the positive control (G2). Due to its strong antioxidant activity and high nutritional content, EBN may have this impact (Jie Cao et al., 2022).

Many investigations on substances originating from plants and animals that are effective against heavy metals have been done (Rana et al., 2018). In the current study, it was anticipated that the action of PbA may lead to the loss or reabsorption of embryo implantation sites between days 4 and 6 post-implantation. Indeed, within the positive control group, the presence of lost embryo implantation sites and reabsorptions was detected. In a previous study, a decrease in the frequency of implanted ova was reported in pregnant animals exposed to lead toxicity at dosage values of 2.2 or 3.0 mg/kg. However, no differences were observed between the experimental (0.5 and 1.5 mg/kg) and control groups regarding the proportion of viable embryos, provided that a normal percentage of ovulating females was considered (Franks et al., 1989). Previous research found that three of the four cycle characteristics were normal during the first exposures and that treated monkeys' cycles were normal before lead exposure. Persistent exposure may disrupt menstruation. Lead-exposed monkeys had more varied intercycle intervals and less vaginal haemorrhages than controls (Strauss et al., 2019). Another study indicated that bone lead can expose target organs during pregnancy, kidney failure, and menopause. In this study, SD mice exposed to PbA had fewer pregnancies and embryos implanted regularly. This trial supported previous studies (Nakade et al., 2015). Enzymes, cytokines, growth factors, and hormones that are involved in uterine physiological processes, including as implantation, may be adversely affected by lead exposure. EBN may contribute to fertility, reproduction, and preventive measures in addition to maintaining health (Filant et al., 2014). PbA exposure has been linked to uterine gland necrosis (Eugenia et al., 2009). Endometrial glands have been connected to oestrous cycles, concepted embryo survival, and development during the peri-implantation stage (Spencer et al., 2004). Studies are being done to see if lead can impair the physiological processes in the uterus (Diantini

et al., 2018; Ogunlade, et al., 2020). EBN supplements stimulated uterine tissue development more than the negative control group and protected against PbA exposure as reported by previous studies (Albishtue et al., 2019). In the current investigation, the groups treated with larger doses of EBN showed enhanced uterine architecture. In a prior investigation, it was demonstrated that lead-induced IGF-1 serum level decreasing decreased the thickness of the uterine wall (Altmäe et al. 2019; Ogunlade, et al., 2020).

PbA disrupts histological structures, prevents follicle growth and development, and increases the proportion of atretic follicles, all of which have been associated to changes in ovarian and uterine functions (He et al., 2020). Because ovarian and uterine rectitude are directly related to fertility, it is crucial to analyse them when evaluating the reproductive system (Quartuccio et al., 2020). By increasing the number of developing follicles and CL and decreasing the number of atretic follicles, EBN has demonstrated favourable effects on the morphology of reproductive organs like ovaries (Albishtue et al., 2018). In the current investigation, degenerative alterations with a considerably larger number of atretic follicles were seen in the ovaries of PbA-treated rats without the addition of EBN ($p < 0.05$), and fewer growing follicles, and CL. According to a study by Jingwen, exposure to PbA may change how the ovary develops, as evidenced by an increase in follicular atresia and a decline in the number of developing follicles (Qu et al., 2021). EBN-supplemented rats, however, displayed resistance against degenerative alterations with a significant ($p < 0.05$) increase in the number of surviving follicles and CL while a decline in number of atretic follicles. Similar to this, EBN has previously demonstrated positive effects on the follicles' maturation and development as well as on the number of follicles that are actively

growing (Albishtue et al., 2018). Infiltration of inflammatory cells, necrosis, and congestion were not significantly different on average ($p < 0.05$) among group G1 and EBN treated groups. However, group G2 had significantly ($p < 0.05$) higher mean pathological lesion scores for inflammatory cell infiltration, follicle degeneration or follicular atresia, and congestion (PbA only). Follicular atresia is a natural ovarian process that regulates the number of follicles in the developing pool. Nevertheless, xenobiotic treatment can also result in an increase in follicular atresia (Sobinoff, et al., 2012). Another PbA exposure model showed that PbA injection to female mice impacted oocyte maturation and follicle layout, resulted in corpus luteum deterioration, and increased the number of atretic follicles (Massányi et al., 2020; Jiang et al., 2021). The ovarian structures of the EBN-treated groups were equivalent to those of group G1 and were normal. As evidenced by a considerably ($p < 0.05$) lower number of atretic follicles in comparison to the G2 group, REBN at doses of 90 and 120 mg/kg and HEBN at a dose of 60 mg/kg both demonstrated protection against cellular adaptive alterations and lesions (PbA only). When the ovary's interstitial cells were scored, it was shown that EBN treatment significantly ($p < 0.05$) boosted their quantity in a dose-dependent way. In comparison to the other treatment groups, the highest value was seen in G4 and G5 with the highest dose of REBN and G8 with the highest dose of HEBN supplement. The interstitial cell count was considerably ($p < 0.05$) lower in group G2 (PbA only) as compared to groups treated with R and HEBN, G1, and G2. EBN's putative bioactive components, which include protein, carbohydrates, ash, and lipids, may be responsible for this impact (Ma & Liu, 2012). Since the main source of carbohydrates in EBN is sialic acid (N. A. Daud et al., 2019). Sialic acid-containing gangliosides have been shown to effectively prevent DNA damage in oocytes, embryos, and other cells from reactive oxygen species (ROS), which is one of the main

causes of PbA toxicity (Kim et al., 2019). EBN has an EGF-like component that has also been linked to EBN's function in adult stem cells' cell division, proliferation, and tissue regeneration (Chua & Zukefli, 2016). Even at the greatest dose, EBN reduced the damaging effects of PbA on ovarian tissues. The results of this study demonstrate that EBN has positive effects on the ovaries in PbA-induced toxicity, which can be attributed to its potential bioactive components and antioxidant function.

When compared to group G1 and the R and HEBN treated groups, the number of uterine glands in group G2 (PbA alone) was considerably lower ($p<0.05$). While there was no discernible difference between the HEBN-treated animals and groups R and G1, there was. Moreover, the (PbA alone) group's destruction of uterine epithelial cells was seen. In group G1, the average score for histological changes such degeneration, congestion, and oedema was not significant ($p<0.05$). As opposed to group G1 and EBN treated groups, the score for infiltration of inflammatory cells (neutrophils), congestion, degradation of LE and GE, and oedema was substantially ($p<0.05$) greater in the PbA alone group. A study conducted by Udayraj et al. (2015) demonstrated that high levels of PbA were found in uterine tissue that displayed histological abnormalities like atrophy, homogeneous endometrial thickening with projections that narrowed the lumen, in addition to ongoing inflammation and endometrial gland destruction (Nakade et al., 2015). In G1 of the current investigation, endometrial atrophy characterized by a decrease in endometrial thickness was also noted. The effects against all histological alterations were greatest with doses of 90 and 120 mg/kg of REBN and 60 mg/kg of HEBN when compared to the rest of the R and HEBN treated groups ($p<0.05$). However, these changes were considerably lower ($p<0.05$) in all EBN treated groups. In the current investigation, groups supplemented with R and

HEBN showed normal uterine morphological characteristics and structures; the number of cells in the LE and glandular structures was larger than in the (PbA alone) group and comparable to that of the negative control group. According to Albishtue et al. (2019), The rat uterus's histomorphological architecture and physiological processes were increased by EBN. As a result, it can be deduced from the fact that PbA's negative effects were reduced when EBN was supplemented concurrently; the greatest protection is attained at REBN doses of 90 and 120 mg/kg bwt and HEBN doses of 60 mg/kg bwt.

Previous investigations have demonstrated that uterine glands in the endometrium manufacture, transport, and secrete chemicals necessary for the conceptus' survival and development (the embryo or foetus and associated extraembryonic membranes) (Filant et al., 2014). Necrosis, a reduction in the number of uterine glands, a reduction in the lining of the uterus' cells, and an increase in the infiltration of inflammatory cells were all seen in PbA-induced rats lacking an EBN supplement, which is consistent with earlier research by (Eugenia et al., 2009). PbA has been demonstrated to cause uterine gland necrosis (Eugenia et al., 2009).

An earlier study found that testosterone and other reproductive hormones are present, E₂, P₄, LH, FSH and prolactin in the EBN (Ma et al., 2012). Oestrogen is thought to be a potential hormone for uterine tissues like epithelium and glandular growth, whereas progesterone is a fundamental hormone for implantation and maintaining pregnancy. These hormones' plasma levels being off can cause issues with fertility and poor reproductive success (Albishtue et al., 2018). The oestrogen and progesterone receptors are primarily found in the cell nucleus, in contrast to certain steroid receptors.

In the current investigation, increasing dosages of EBN led to greater PR expressions in GE and stoma cells. Throughout the window of implantation, stroma cells and GE showed an elevated impression of PR (Albishtue et al., 2019).

In addition to guarding against the negative effects of PbA, a 60mg/kg dose of HEBN supplement may have also encouraged the growth of uterine structures more than the negative control group (with no EBN and PbA treatment). The findings of this investigation are in agreement with those of earlier studies, which demonstrate a decrease in LE thickness as a result of lead-induced suppression of IGF-1 serum levels (Diantini et al., 2018, Altmä et al., 2019, Ogunlade et al., 2020). IGF-1 action may also result in a rise in uterine epithelial thickness (Altmä et al., 2019, Ogunlade et al., 2020). The current investigation of HEBN supplement on the uterine and glandular epithelium and stromal cells showed a dose-dependent effect of 60 mg/kg bwt on the growth factors expressed on uterine and glandular epithelium as well as stromal cells. Pinopodes are generated from the uterine luminal epithelium's apical surface during the window of receptivity in both rodents and humans, and they can only be observed with SEM. Pinopodes are frequently used to evaluate endometrial receptivity (Quinn and Casper, 2009). When compared to the control and PbA exposure groups in our study, SEM results revealed pinopods and microvilli in the uterine luminal epithelium in G8 (60mg/kg HEBN). The EGF-like component in HEBN has been associated with cell division and tissue regeneration (Choy et al., 2021) and its role in HEBN (Quek et al., 2018). The results of this study show a significant relationship between elevated EGF expression and ovarian stromal and epithelial cell proliferation. As it directly influences the proliferation of human adipose-derived stem cells, macrophage cell lines, and human colonic cancer cell lines, HEBN has the capacity to boost mitogenic responses and give viability (Quek et al., 2018).

At steady condition, more than 90% of the Pb body burden is found in the skeleton. Between 50 and 75 percent of blood Pb in young adult women comes from tissue reserves (Grant, 2020). Smith et al. (1996), stating that 40–70% of the lead in blood came from the skeleton. Pb that has been absorbed from blood plasma is dispersed to various organs, with liver and kidney having the largest quantities. According to a study by Abubakar et al. (2019) hepatocytes in the treated group revealed considerable vacuolation, pyknotic hepatocytes with a clogged central vein, and the presence of Kupffer cells in the structures of the liver tissue (Abubakar et al., 2019). In a different study, lead treatment groups received 50 mg of lead daily for 4 weeks, which caused focal liver tissue necrosis, vacuolation of hepatocytes, activation of Kupffer cells, clogged blood arteries, and neutrophil infiltration (Abubakar et al., 2019). Similar results were seen in the current investigation, where G2 (the positive control group) showed considerably higher levels of hepatocyte vacuolation, tissue necrosis as well as activation of Kupffer cells as compared to the control, other R, and HEBN treated groups. Yet, these modifications were significantly smaller ($p < 0.05$) In all H and REBN treated groups, the doses of 120 mg/kg REBN and 60 mg/kg HEBN had the greatest impact on all histological changes. Hepatocytes were seen in higher numbers than in G1 (the control group) and were comparable to those of the negative control groups in the groups supplemented with EBN in the current investigation. According to Albishtue et al. (2019), The rat kidney's histomorphological architecture and physiological activities were increased by EBN. Hence, it can be inferred from the aforementioned result that Pba's negative effects were reduced by concomitantly supplementing H and R EBN; the greatest protection is obtained with REBN doses of 90 and 120 mg/kg bwt and HEBN doses of 60 mg/kg bwt.

Abubakar et al. (2019) found that lead treatment at 50 mg/kg for 4 weeks enlarged Bowman's capsule and atrophy glomeruli. Inflammatory cells, renal tubule necrosis, and blocked blood arteries were also detected (Abubakar et al., 2019). The lead-treated group got 10 mg/kg daily for 8 weeks, and histological analysis of the kidneys showed necrosis, glomerulus atrophy with damaged bowman's capsule, and inflammatory cell infiltration. However, these modifications were significantly decreased ($p < 0.05$) in all H and REBN-treated groups, and 120 mg/kg REBN and 60 mg/kg HEBN were most effective against all histological alterations. In the current study, EBN-supplemented groups had normal parenchymal structures and morphological characteristics, glomerulus cells, and an intact bowman's capsule structure. According to Albishtue et al. (2020), EBN improved rat kidney histomorphology and function. Thus, concomitantly supplementing H and R EBN reduced Pba's deleterious effects. REBN doses of 90 and 120 mg/kg bwt and HEBN doses of 60 mg/kg bwt provided the highest protection. Reproductive organ histology confirmed EBN's protection. Raw and hydrolyzed EBN reduced lead acetate-induced tissue damage and cell abnormalities. EBN may protect reproductive organs against lead acetate. Hydrolyzed EBN protected better than raw. Bioactive peptides released by hydrolyzed EBN may have increased its protective properties.

The results of this study add to our understanding of the probable processes underlying EBN's reproductive system protection from lead acetate toxicity. EBN's antioxidant, anti-inflammatory, immunomodulatory, and heavy metal chelating qualities are anticipated to play major roles in its protective benefits. The oxidative stress, inflammation, and disruption of reproductive processes brought on by lead acetate may be lessened by these qualities.

6.5 Conclusion

The current comparative analysis confirms PbA's harmful effects on the reproductive system and shows that oral supplementation with Red (R) and House (H) edible bird's nest (EBN) protects pregnant rats from PbA-induced reproductive toxicity and organ damage. The highest fertility, endometrial receptivity, embryonic implantation rate, and uterine epithelium microvilli with pinopodes were achieved after eight weeks of supplementation with 90 and 120 mg/kg R EBN and 60 mg/kg H EBN. These dosages increased interstitial cells and corpus lutea (CL) and decreased ovarian atretic follicles. Histology showed thicker, bigger uterine glands and taller columnar cells in epithelium. Liver tissues had typical central veins and binucleated hepatocytes, while kidney tissues had intact Bowman's capsules and glomeruli, especially with medium to high R EBN and H EBN dosages. Uterine tissues expressed more steroid receptors EGF, EGFR, PCNA, and VEGF. Immunohistochemical analysis of the ovarian surface epithelium and interstitial cells in the medium and maximal dosage groups of R and H EBN showed considerable steroid hormone, EGFR, VEGF, and PCNA expressions. ICP-MS showed that EBN supplementation reduces PbA deposition in both tissues dose-dependently. The positive control group also demonstrated increased immunohistochemical expressions of metallothionein (MT) in uterine, ovarian, kidney, and liver tissues. The positive control group had higher TBARS and lower SOD levels than the treatment groups, indicating a greater disturbance of redox equilibrium and oxidative stress. Thus, the full potential of R and H EBN would not only advance the development and use of efficient fertility measures but also provide empirical evidence to support the widely held belief that they improve mammalian reproduction.

CHAPTER 7

EFFECTS OF RAW AND HYDROLYZED EBN ON EXPRESSIONS OF GROWTH FACTORS, STERIOD HORMONES AND MT, REDOX LEVEL, AND ACCUMULATION OF LEAD IN REPRODUCTIVE TISSUES OF RATS EXPOSED TO LEAD TOXICITY

7.1 Introduction

Lead acetate harms various physiological systems, including the reproductive system. EBN, made from swiftlet cave dwellers' saliva, may protect against lead acetate toxicity. Raw and hydrolysed EBN protects rat uterine, ovarian, renal, and hepatic tissues, however the mechanism is unknown. Lead acetate poisoning can cause ovarian and uterine dysfunction. Lead intoxication can also harm liver and kidneys. Understanding how EBN protects these tissues may help us employ it therapeutically.

Lead (Pb) is one of the most important naturally occurring hazardous heavy metals found in the Earth's crust. (Rehman et al., 2021). Lead originated from Latin word plumbum (Boldyrev, 2018). Pb pollutes all living things and the environment. Many heavy metals and compounds are hazardous to mammalian reproduction (Sofoulaki et al., 2019; Karri et al., 2020). Lead impairs mammalian reproduction through biochemical, behavioural, and physiological impacts. Metal ore dusts, Pb-battery manufacture, and mining-contaminated food and water are major sources of lead toxicity. Pb was found in the endocrine glands of Pb-exposed personnel and animal models by Mitra et al. (2017). Several studies found harmful effects on the cardiovascular system, central nervous system, haematopoiesis, kidneys, liver, and reproductive system (Singh et al., 2013, Renieri et al., 2019). Pb attracts protein

sulfhydryl groups, essential to many biological activities. Pb binding to these groups disrupts protein function and damages cells. Pb can mimic or block calcium, an essential ion for muscle contraction and nerve signal transmission, can be absorbed by cells and accumulate in tissues, disrupting calcium transmission and causing toxicity (Kościuk et al., 2019). Pb inhibits growth hormone (GH), decreasing somatotrope responsiveness and severely damaging the hypothalamic-pituitary axis. Lead, cadmium, and arsenic can influence hormones, reproductive difficulties, and infertility in women (Ahmed et al., 2013, Shojaeepour et al., 2018, Singh et al., 2018).

PbA has been labeled as a dangerous chemical because it accumulates in animal tissues and has a very long half-life (Rzymiski et al., 2014). Women with 10 µg/dL BPb level face complications like preterm birth, low birth weight, infertility, miscarriage, neural defects in early childhood, and maternal hypertension (Van et al, 2000). Pb exposure reduced rats' reproductive organs' sodium potassium ATPase and alkaline phosphatase (ALP) enzyme activity, lowering fertility. Pb exposure induces lipid peroxidation in liver tissues (Shafiq-ur-Rehman., 1984, Benoff, S. et al., 2000). OS, enzyme inhibition, DNA damage, gene expression, and adventitious mimicry are multifactorial mechanisms. Pb-mediated OS damages cell membranes, DNA, enzymes like catalase, GPx, SOD, and glucose-6-phosphate dehydrogenase (G6PD), and non-enzymatic antioxidant compounds such thiols (GSH) in mammalian systems (Valko et al., 2005; Flora et al., 2008). In the signaling system, Pb mimics calcium and binds with calmodulin protein, which induces lead poisoning. Phyto-antioxidants (Catechin, Carotenoids, β-carotene, Flavonoids, Garlic, Lipoic acid, Lutein, Lycopene, and Quercetin) are polyphenolic compounds that form a complex with lead and break the chain reaction to reduce or prevent OS (Terao et al., 2009). Herbal antioxidants such as ginkgo and curcumin have been reported to protect against lead induced OS (Singh

et al., 2018). EBN, a naturally occurring substance, similarly reduces Pb toxicity in reproductive system histomorphology and function by preserving antioxidant–ROS equilibrium (Albishtue et al., 2019). It also prevents PbA-induced reproductive harm. PbA poisoning can damage fertility, thus it's important to study its effects and investigate natural substances that may improve fertility. PbA can be mitigated in several ways (Kościuk et al., 2019). EBN's anti-inflammatory, antioxidant, bone-strengthening, and fertility-enhancing properties may help attenuate heavy metals' impacts on mammalian reproduction and fertility (Choy et al., 2021). EBN's growth factors—IL-6, VEGF, and EGF—have been proved to be advantageous. EBN EGF increases cell division and tissue regeneration. IL-6 and VEGF regulate inflammation and angiogenesis in EBN. VEGF promotes blood vessel formation, while IL-6 is a pro-inflammatory cytokine implicated in the immunological response. Growth factors help EBN rejuvenate (Kong et al., 1989).

Conjugated sialic acid and N-acetylneuraminic acid in EBN are immunomodulatory, antioxidant, and anti-inflammatory. EBN often contains indigestible bioactive components. Enzymatic hydrolysis can simplify these complex chemicals so the body can absorb them. This may boost bioavailability and therapeutic potential of bioactive components (Wong et al., 2018). A prior investigation by Albishtue et al. (2019), showed that PbA affected male rats upon treatment with EBN showed significant improvement in their sperm count, motility, and viability, and showed reduced morphological abnormalities. This study compared Raw and Hydrolyzed Edible Bird's Nest (REBN and HEBN) for PbA mitigation. EGF, EGFR, VEGF, PCNA, and steroid hormone receptors were examined to determine REBN and HEBN's effects on reproductive tissue growth and development. This study examined REBN and HEBN therapy's antioxidant and oxidative stress indicators. ICP-MS was utilized to measure REBN and HEBN reproductive tissue PbA toxicity.

7.2 Materials and methods

7.2.1 EBN and Lead acetate preparation

The preparation of R and H EBN and lead acetate was carried out in the same manner as stated in Chapter 3.

7.2.2 Experimental design

Female Sprague-Dawley rats reached sexual maturity at 12 weeks of age, at which point they were housed in groups of 6 (8 cages total). The rats were acclimated for 7 days before being randomly divided into 8 groups (6 animals/group) and exposed to Lead Acetate (PbA) for 8 weeks while receiving in vivo supplements of R & H EBN as shown in Table 7.1 below. A previous study was used to guide the selection of the R and H EBN dose (Albishtue et al., 2019). Histological studies and degree of expressions of EGF, EGFR, VEGF, PCNA, MT, ER and PR of uterine, ovary tissues, along with histological studies and level of expressions of MT of liver and kidney tissues were carried out as described in Chapter 6.

Table 7.1: Animal grouping and treatment

Group	Group assigned	Type of treatment (dose)/8 weeks
Control	G1 (Control)	Normal diet +Distill water
Treatment Group	G2 (Positive control)	PbA only (10mg/kg)
	G3 (Low dose)	PbA + REBN (10mg/kg + 60mg/kg bwt.)
	G4 (Medium dose)	PbA + REBN (10mg/kg + 90mg/kg bwt.)
	G5(High dose)	PbA + REBN (10mg/kg + 120mg/kg bwt.)
	G6 (Low dose)	PbA + HEBN (10mg/kg + 20mg/kg bwt.)
	G7 (Medium dose)	PbA + HEBN (10mg/kg + 40mg/kg bwt.)
	G8 (High dose)	PbA + HEBN (10mg/kg + 60mg/kg bwt.)

ND=Normal diet, PbA=Lead Acetate, REBN= Raw edible bird's nest, HEBN= Hydrolysed edible bird's nest, bwt=Body weight.

7.2.3 Conformation of oestrous cycle phases and the oestrous synchronization

Oestrous cycle phases and the oestrous synchronization determination were carried out in the manner outlined in Chapter 3.

7.2.4 Assessment of expressions of EGF, EGFR, PCNA, VEGF, MT, E2R, and P4R in the uterine and the ovarian tissues by IHC

EGF, EGFR, PCNA, VEGF, MT, E2R, and P4R expressions were evaluated in uterine and ovarian tissues by IHC as reported in Chapter 3.

7.2.5 Effect of R and HEBN on tissue level PbA analysis using coupled plasma spectrometry (ICP-MS)

Immunohistochemistry (IHC) was used to examine the expression of metallothionein. Tissue sections (4 μ m) were collected from the paraffin-embedded chunks and placed on the new glass slides, where they were let 15 hours to dry by air. A series of xylene and alcohol solutions were used to deparaffinize the slides. The sections were submerged in diluted target retrieval solution (pH 6.0) for a predetermined period of time to retrieve the antigen (Dako, North America). After being heated on high power for 10 minutes, the container containing the retrieval solution, and the submerged slides was allowed to cool naturally to room temperature. After discarding the Target Retrieval Solution (TBS), the sections were washed three times in tris buffered solution. The tissue was marked with a Dako pen after the excess wash buffer was tapped off (Dako, Denmark). An IHC detection kit (Code ab236469, Abcam USA) was used to heat-inactivate endogenous peroxidase in the sample for 10 minutes at 37 °C. Samples were TBS-washed. To prevent endogenous immunoglobulins from

reacting with the secondary antibody, the sample was incubated with the protein block at 37 °C for 10 minutes in TBS. After that, samples were incubated at 4 °C for 30 minutes with the rabbit polyclonal anti-Metlothionein antibody ab192385, diluted 1:150, from Abcam, USA. Lacking the primary antibodies, negative controls were created. TBS was used to clean the samples before the Peroxidase-labelled polymer secondary antibody, IHC detection kit was applied for 15 minutes at 37°C (Code ab236469, Abcam USA). The slides were developed using the chromogen DAB for 10 minutes (3, 30-diaminobenzidine). Rinse the stain with clean water to get rid of it. I counterstained the slides by submerging them in a haematoxylin wash for three minutes. Based on the degree of the immunostaining, the expression of metallothionein in different LE and GE cells was graded and divided into four categories: 0 denotes absence, A score of 1 indicates a weak positive effect, 2 a moderate one, and 3 a substantial one. Each sample included five parts and five fields chosen at random. We counted and ranked 100 LE cells and 100 GE cells from each sample. The following equation was used to determine the immunohistochemistry score. The Medical Image Analysis microscope (Motic Image plus 2.0, China) was used to examine an additional five slices and five fields from each sample (Albishtue et al., 2019).

7.2.5.1 Chemicals and reagents

The ultrapure water used to create the reagents and standards was measured at 18.2 MW cm@25 °C (MilliQ® Integral 3, Millipore-Merck, Darmstadt, Germany). All glassware was treated with 5% (v/v) HNO₃ for 24 hours prior to analysis. Ultrapure water was used three times to clean it before it was put away to dry. For trace analysis, Sigma Aldrich in the United States used HNO₃ and HCl. An ICP-MS tuning solution

containing 10 mg/L each of lithium, yttrium, cerium, thallium, and cobalt was developed by Agilent Technologies in Barcelona, Spain. This 0.1 ppm internal standard solution was developed by Agilent Technologies to account for instrument drifts and matrix effects. Multi-element calibration standard 2A (Agilent Technologies, Barcelona, Spain), diluted to NIST traceability, was used to create external Plumbum (Pb) calibration standards. A blank solution was made by dissolving 2% nitric acid (HNO₃) and 5% hydrochloric acid (HCl) in ultrapure water. From 1 to 100 ppb, external calibration curves were developed.

7.2.5.2 Sample preparation

A portion of the samples (1.0 g) were digested in an ETHOS microwave digestion machine (Milestone, Gomensoro, Barcelona, Spain) with 8 ml of nitric acid for trace analysis (HNO₃). The digestion process went like this: 15 minutes at 220 °C and 800 watts of maximum power. The digested sample was then diluted to a level of 100mL with ultrapure water.

7.2.5.3 Sample analysis with ICP-MS

Using an Agilent 7700x inductively coupled plasma mass spectrometer and a Micro Mist nebulizer (Agilent Technologies, Barcelona, Spain), plumbum (Pb) was measured (Glass Expansion, Melbourne, Australia). Table 3.5 displays the characteristics and usage guidelines for the Agilent 7700x ICPMS. External calibration standards were used to quantify the results. Every sample was digested and analysed twice. QC was carried out by analysing 20 ppb of calibration standard for every 8 samples (Figure 3.5).



Figure 7.1: Procedures of ICP-MS for the analysis of PbA levels in uterine tissue

Table 7.2: Agilent 7700x ICPMS parameters and operating conditions

Name	Value
RF Power	1550 W
RF Matching	1.6V
Sample depth	9.5 mm
Torch	-0.1 mm
Torch-V	0.0 mm
Argon Gas Flow Rate	15L/min
Carrier Gas Flow	1.01 L/min
Make up Gas Flow	0.15 L/min
Auxiliary Gas Flow Rate	0.58mL/min
Sample Uptake Rate	0.3 rps, 100 µL/ min
Sampling Depth	6 mm ~ 7.6 mm
Spray chamber temperature	2° C
Nebulizer Pump	0.1 rps
Integration Time	3 s
Internal Standard	²⁰⁹ Bi

7.2.6 Effect of R and HEBN on SOD and TBARS levels

Analyses of the plasma concentration of SOD and TBARS were completed as described in Chapter 3.

7.2.7 Statistical analysis

Graph Pad Prism 8.0 calculated the mean (M) and standard error of the mean (SEM). Graph Pad Software, San Diego. The ratio of ovarian to uterine bwt, SOD levels, PbA levels in uterine tissues, and MT gene expression were assessed using a one-way ANOVA and Tukey multiple comparison post-hoc test. To analyse body weight gain two-way ANOVA and Bonferroni's multiple comparison testing were applied. P values of 0.05 indicated statistical significance.

7.3 Results

7.3.1 Uterine examinations using scanning electron microscope (SEM)

At Pd8 uterine tissues were observed under scanning electron microscope from control and EBN-supplemented (REBN & HEBN), smooth folds and pinopods were observed in the uterine epithelium at embryo implantation sites (Figure 7.1). In G2 and 60mg/kg (G3 group), showed fewer folds along with irregular pinopods. Microscopy of the uterus revealed smooth folds with small microvilli and smooth large pinopods in negative control (G1 group). Higher dose treatments at G4: REBN 90 mg/kg bwt; G5: REBN 120 mg/kg bwt showed significantly ($p<0.05$) more microvilli and extensive pinopods compared other EBN treatment groups, negative control (G1) and positive control (G2) group (Figure 7.1).

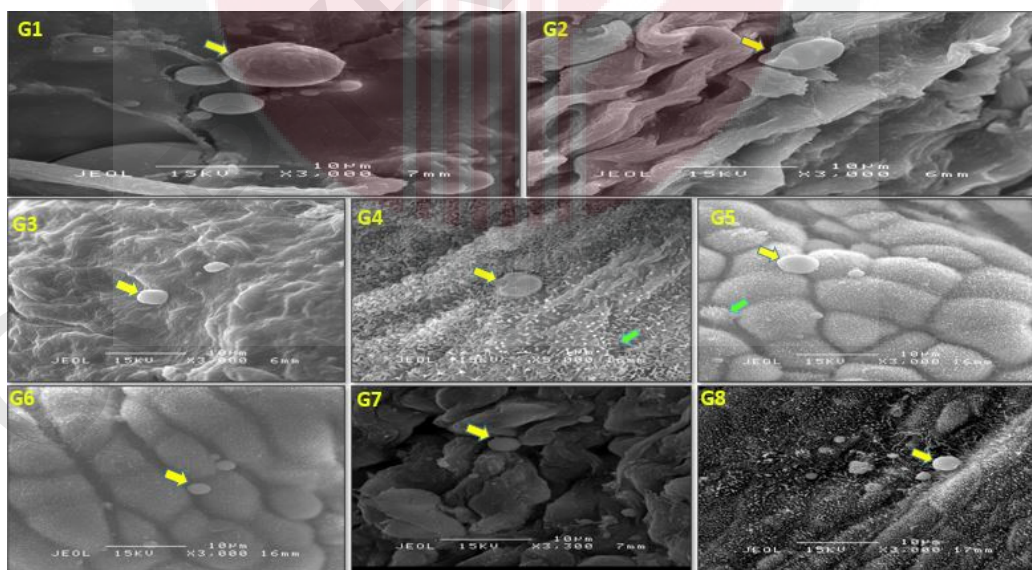


Figure 7.2: Scanning electron micrograph of the R and HEBN treated groups when compared to the control and PbA group

*G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). Note, Yellow arrow shows pinopods while green arrows show microvilli on Luminal epithelium (LE) and Glandular epithelium (GE) of uterus.

7.3.2 Expressions and scoring of EGF and EGFR in the uterine tissues

Figures 7.2 and 7.3 show immunohistochemical photomicrographs of EGF and EGFR expressions in uterine tissue slices from all experimental groups, while their relative score differences are indicated in Table 7.1. The density of stromal (S) cells, as well as EGF and EGFR expression in the luminal and glandular epithelium (LE and GE), of the gravid uterus were all boosted by EBN therapy. Most measures improved with increasing dose. Figure 7.2 shows that EGF expression is apparent in LE, with stronger staining in G5 (REBN 120 mg/kg bwt) and G8 (HEBN 60 mg/kg) compared to controls and other EBN-treated groups. Gene expression was shown to be considerably upregulated ($p < 0.05$) in the EBN treatment groups G5 and G8 receiving the highest dosages of REBN 120 mg/kg bwt and HEBN 60 mg/kg bwt, respectively. EGF, and EGFR, expressions in G5 (REBN of 120 mg/kg bwt) and G8 (HEBN of 60 mg/kg bwt) were observed significantly higher ($p > 0.05$) when compared to other EBN treated groups and control. As shown in Table 7.1 (Figures 7.2-7.3), the levels of expression were much higher in S, GE, and LE cells than they were in control and G2 cells. Despite the fact that EGF, EGFR, the dose of EBN supplemented increased the uterine tissues' expressions, but the strength and structure of the expressions varied by tissue and cell location (Figures 7.2-7.3). In the control group, a few S, GE, and LE cells with partial nucleus and cell membrane staining started EGF expression. In G5 (REBN 120 mg/kg bwt) and G8 (HEBN 60 mg/kg bwt), EGF expression increased from modest to strong staining throughout all areas when compared to negative and positive control and other R and H EBN treated groups. (Figure-7.2). For both G5 and G8, EGF expression was found to be present at significantly ($p > 0.05$) high levels in the nucleus of most S, GE and LE cells. In G5 (REBN of 120 mg/kg bwt) and G8 (HEBN of 60 mg/kg bwt) groups, the immune response was denser and more robust at the GE.

Figures 7.3 reveal that a very less distinct immune reaction was discovered in G1, G2, G3, G4, G6, and G7 slides for EGFR. The scenario for EGFR was slightly different. The EGFR response in G4 and G5 (REBN of 90 and 120 mg/kg bwt) and G8 (HEBN of 60 mg/kg bwt) groups cells is substantial, although a significant fraction of S, GE, and LE cells have no EGFR signalling in their cytoplasm. Expression levels were observed significantly ($p > 0.05$) higher in EGFR expression in G4 and G5 (REBN of 90 and 120 mg/kg bwt) and G8 (HEBN of 60 mg/kg bwt) when compared to control and other R and HEBN treated groups (Figure 7.3).



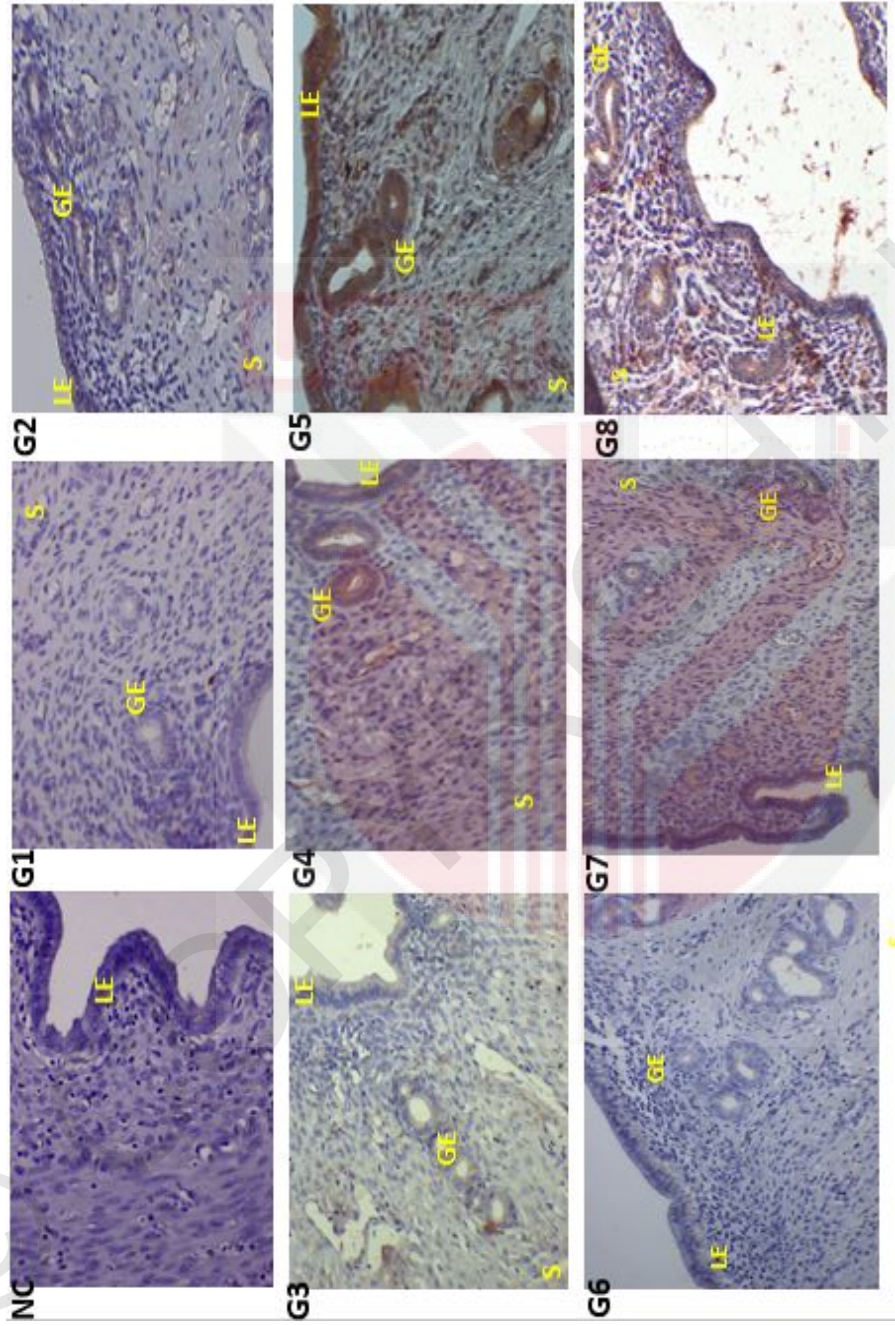


Figure 7.3: Photomicrograph of uterine tissues of different experimental groups treated with PbA and different doses of R and HEBN showing EGF expression

* NC: without primary antibodies. G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). EGF immunoreactivity is evident in LE, staining intensity was higher in G4, G5 and G8 when compared to Control and R and HEBN treated groups. Magnification 200x.

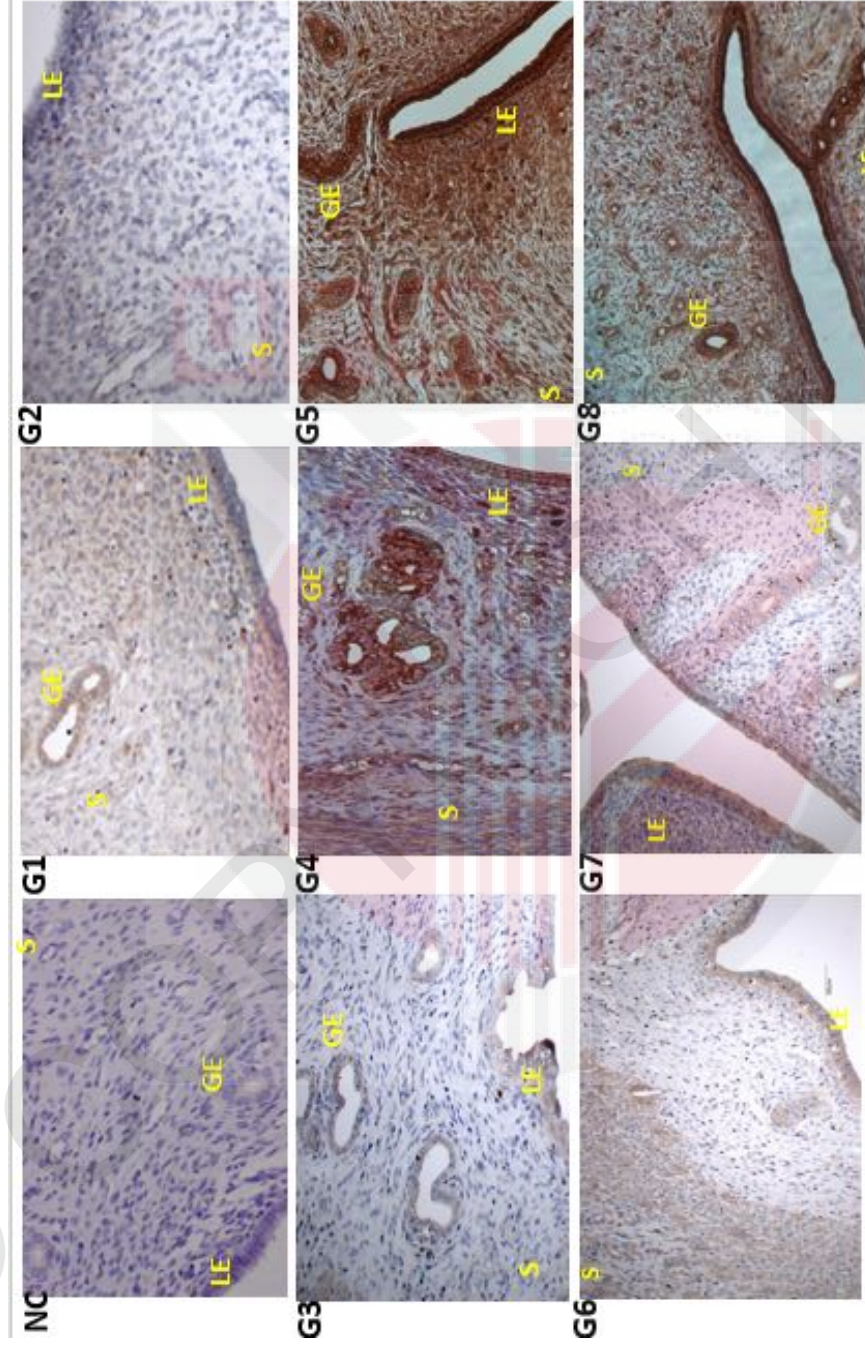


Figure 7.4: Photomicrograph of uterine tissues different experimental groups treated with PbA and different doses of R and HEBN showing EGFR expression
 *NC: without primary antibodies. G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) and G5= (REBN 90 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). EGFR immunoreactivity is evident in LE, staining intensity was higher in G4, G5 and G8 when compared to control and R and HEBN treated groups. Magnification 200x.

Table 7.3: Effect of different levels of raw and hydrolysed edible bird's nest fed to lead acetate (PbA)-exposed rats on the score in the LE, GE, and S cells of uterine tissues of rats

Parameters	Groups							
	G1	G2	G3	G4	G5	G6	G7	G8
EGF in LE	2±1 ^b	1±0 ^c	1.3±0.3 ^b	2.1±0.16 ^a	3±0 ^a	0.5±0 ^c	1.6±0.8 ^a	3±0 ^a
EGF in GE	2±0.5 ^b	0±0 ^c	0±0 ^c	2.3±0.6 ^a	3±0 ^a	0.5±0 ^c	2±0 ^b	3±0 ^a
EGF in S	1.6±0.8 ^b	0±0 ^c	0±0 ^c	1.3±0.3 ^b	3±0 ^a	0±0 ^c	1±0 ^c	2.5±0 ^a
EGFR in LE	1.3±0.16 ^b	0±0 ^c	0.3±1.6 ^c	2.3±0.3 ^a	3±0 ^a	1±0 ^c	2.5±0 ^a	3±0 ^a
EGFR in GE	2±0.5 ^a	0±0 ^c	0±0 ^c	2.8±0.16 ^a	3±0 ^a	1±0 ^c	1.5±0 ^c	3±0 ^a
EGFR in S	1.3±0.3 ^b	0.3±0.16 ^c	0±0 ^c	1.8±0.16 ^b	3±0 ^a	1.5±0 ^b	0.5±0 ^b	2.8±0.16 ^a

Means with different superscripts within each row differ significantly (P<0.05).

*G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) G4= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). LE = uterine luminal epithelium; GE = uterine glandular epithelium; S = stromal cells.

7.3.3 Expressions and scoring of VEGF and PCNA in the uterine tissues

Figures 7.4-7.5, show VEGF and PCNA immunohistochemistry photomicrographs of uterine tissue slices from all experimental groups while their comparative score differences are indicated in Table 7.2. EBN treatment increased the density of luminal, glandular, and stromal (S) cell VEGF and PCNA expression in the gravid uterus. Most parameters increased dose-dependently. As shown in Table 7.2 (Figures 7.4-7.5), the levels of expression were much higher in S, GE, and LE cells of medium to higher EBN dose treated groups when compared to lower doses of EBN, control and G2 cells. At 90 mg/kg bwt of REBN supplement (G4), All regions of the S, GE, and LE showed signs of VEGF responses, with expression and staining that was identical to that seen in groups G5 (REBN of 120 mg/kg bwt) and G8 (HEBN of 60 mg/kg bwt) ($p > 0.05$) (Figure-7.4). A detailed examination of multiple slides revealed a mild reaction in PCNA for G1 (control) and G2 (positive control) (Figures 7.5) It showed only barely perceptible reactions along the S and LE. The staining reaction for EGF, however, went from being weak in the beginning of the sections to being very strong towards the significantly ($p > 0.05$) higher in G5 (REBN of 120 mg/kg bwt) and G8 (HEBN of 60 mg/kg bwt) groups were shown to have PCNA expression in all locations of the S, GE, and LE (Figure 7.5). Results of IHC expressions in uterine surface epithelium and uterine stromal cells showed significantly higher ($P < 0.05$) expressions of steroid receptors, PCNA, and VEGF in group G4 (REBN 90 mg/kg bwt), G5 (REBN of 120 mg/kg bwt) and G8 (HEBN of 60 mg/kg bwt) when compared to other R and H EBN treated groups and with G1 and G2.

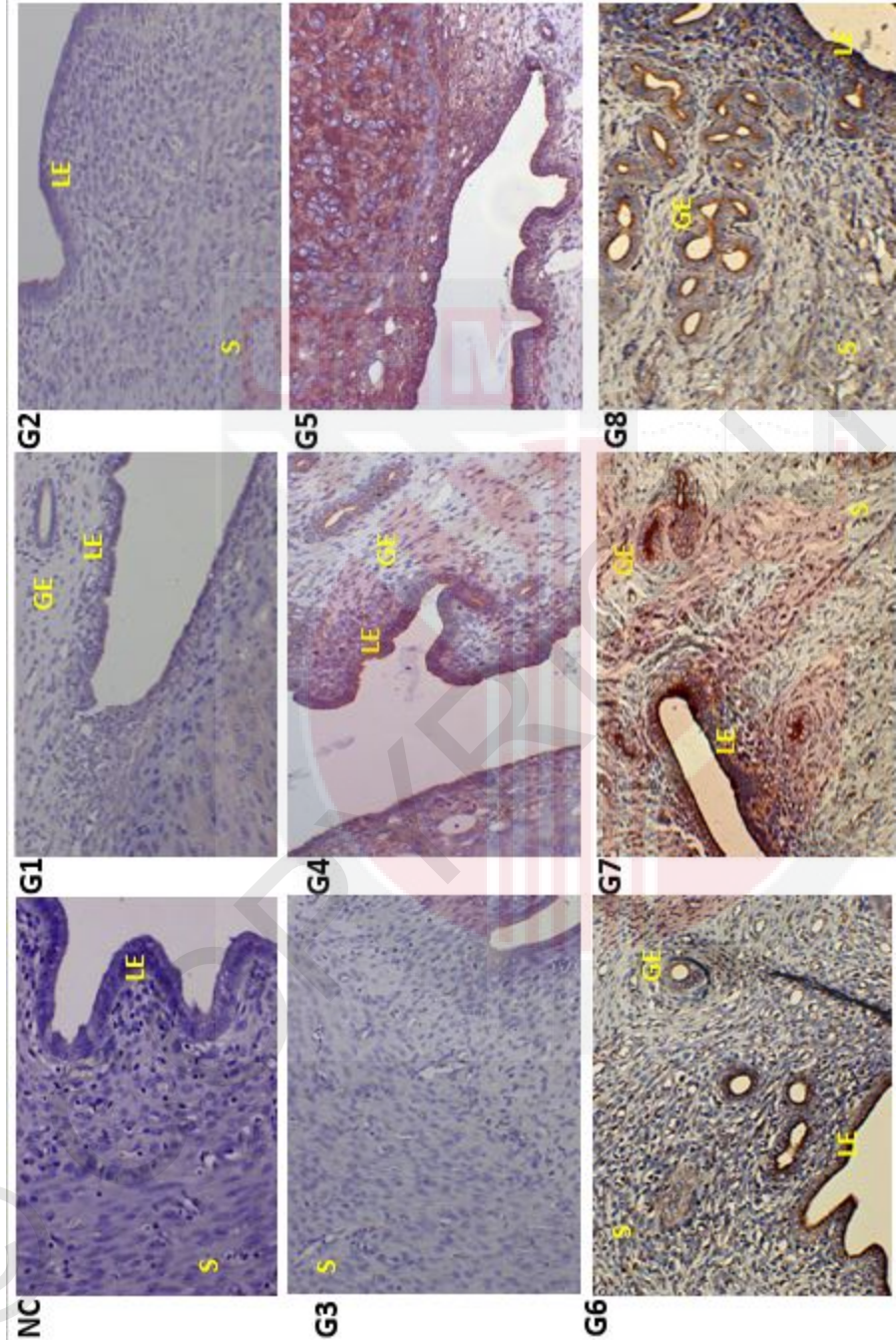


Figure 7.5: Photomicrograph of uterine tissues different experimental groups treated with PbA and different doses of R and HEBN showing VEGF expression
 *NC: without primary antibodies. *G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). VEGF immunoreactivity is evident in LE, staining intensity was higher in G5 and G8 as compared to control and R and HEBN treated groups. Magnification 200x.

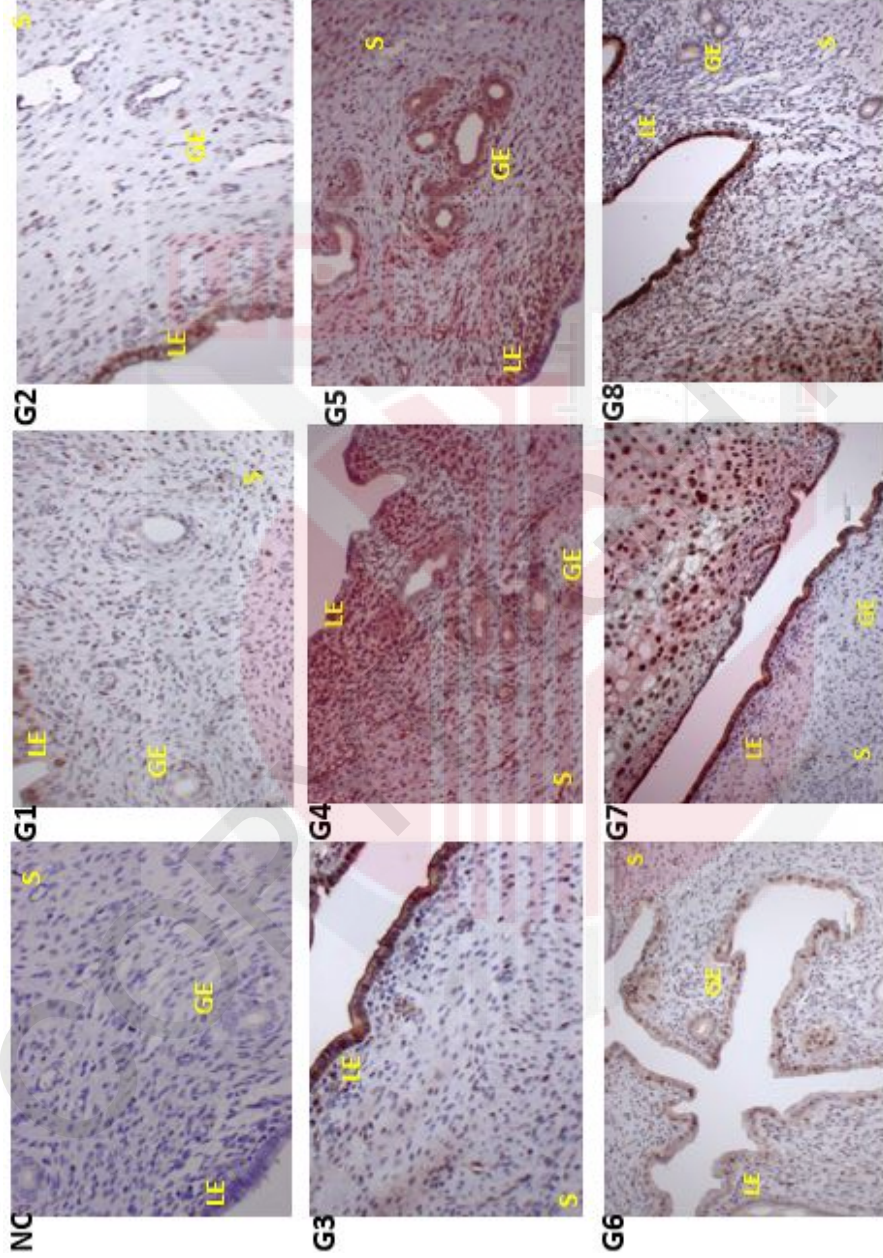


Figure 7.6: Photomicrograph of uterine tissues different experimental groups treated with PbA and different doses of R and HEBN showing PCNA expression

*NC: without primary antibodies. G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). PCNA immunoreactivity is evident in LE, staining intensity was higher in G4, G5 and G8 when compared to control and R and HEBN treated groups. Magnification 200x

Table 7.4: Effect of different levels of row and hydrolysed edible bird's nest fed to lead acetate (PbA)-exposed rats on the score VEGF and PCNA in the LE, GE and S cells of uterine tissues of rats

Parameters	Groups							
	G1	G2	G3	G4	G5	G6	G7	G8
VEGF in LE	1.6±0.16 ^b	0±0 ^c	0±0 ^c	1.5±0.28 ^c	2.5±0.28 ^a	1.16±0.12 ^b	2.5±0 ^a	2.5±0 ^a
VEGF in GE	1.8±0.16 ^b	0±0 ^c	0±0 ^c	2±0 ^b	2.6±0.16 ^a	2.5±0 ^a	2±0 ^b	2.3±0.1 ^a
VEGF in S	1.6±0.3 ^b	0±0 ^c	0±0 ^c	1.3±0.3 ^a	1.6±0.3 ^b	2.5±0 ^a	2.5±0 ^a	2.6±0.1 ^a
PCNA in LE	2.5±0.28 ^a	1±0 ^c	2±0 ^a	1.6±0.8 ^b	2.8±0.16 ^a	1.5±0 ^b	3±0 ^a	3±0 ^a
PCNA in GE	1.6±0.3 ^b	0.3±0.3 ^c	0±0 ^c	2.3±0.6 ^a	2.6±0.16 ^a	2±0 ^a	2.3±0 ^a	2.5±0 ^a
PCNA in S	1.3±0.16 ^c	0±0 ^c	0±0 ^c	2.1±0.16 ^a	3±0 ^a	1±0 ^c	1±0 ^c	3±0 ^a

Means with different superscripts within each row differ significantly (P<0.05).

*G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) G4= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). LE = uterine luminal epithelium; GE = uterine glandular epithelium; S = stromal cells.

7.3.4 Assessment of steroid hormone receptors, E2R and P4R in uterine tissues

Figures 7.6 and 7.7 show immunohistochemical photomicrographs of E2R and P4R expressions in the uterine tissue sections of all treated groups, while the related score variations are indicated in Table-7.3. Stimulated E2R expression and enhanced stromal cell density were seen in the S, GE, and LE when the EBN dose was raised. E2R expression was significantly ($p<0.05$) higher in the rats supplemented with the highest dose of REBN of 120 mg/kg bwt (G5) than the control and other R and H EBN treated groups (Figures 7.6). Other groups showed the second greatest E2R expression, which was also considerably higher than the negative control and the positive control that did not show any specific reaction (Figures 7.6). However, a P4R antibody was used to label the nuclei of stromal cells and GE. Group G5 (REBN of 120 mg/kg bwt) and Group G8 (HEBN of 60 mg/kg bwt) showed significantly higher P4R expressions ($p<0.05$; Figures 7.7), while Groups G1, G2, G6, and G7 showed significantly lower expressions.

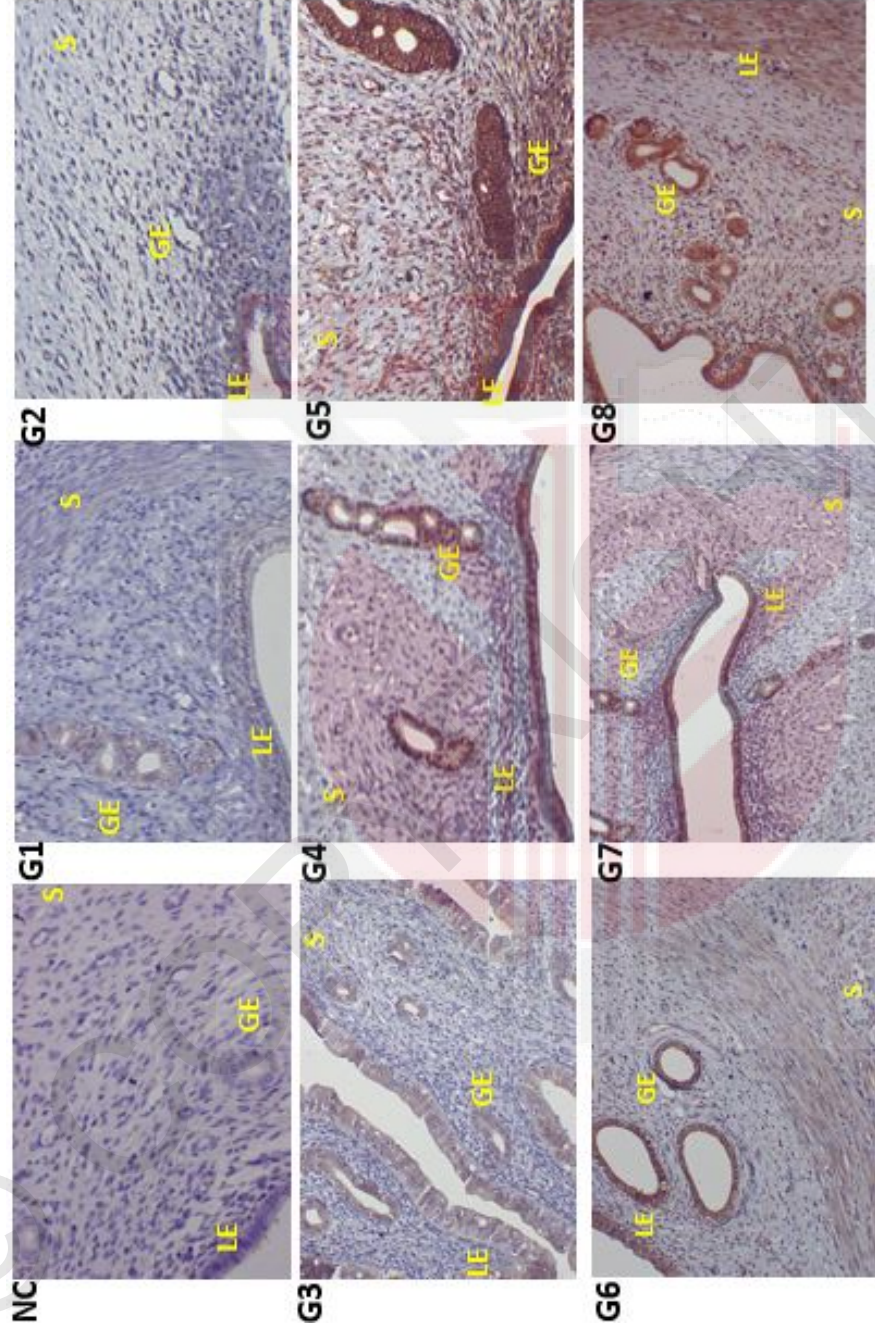


Figure 7.7: Photomicrograph of uterine tissues different experimental groups treated with PbA and different doses of R and HEBN showing E2R expression

*NC: without primary antibodies. G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). ER immunoreactivity is evident in LE and GE, staining intensity was higher in G5 and G8 when compared to control and R and HEBN treated groups. Magnification 200x.

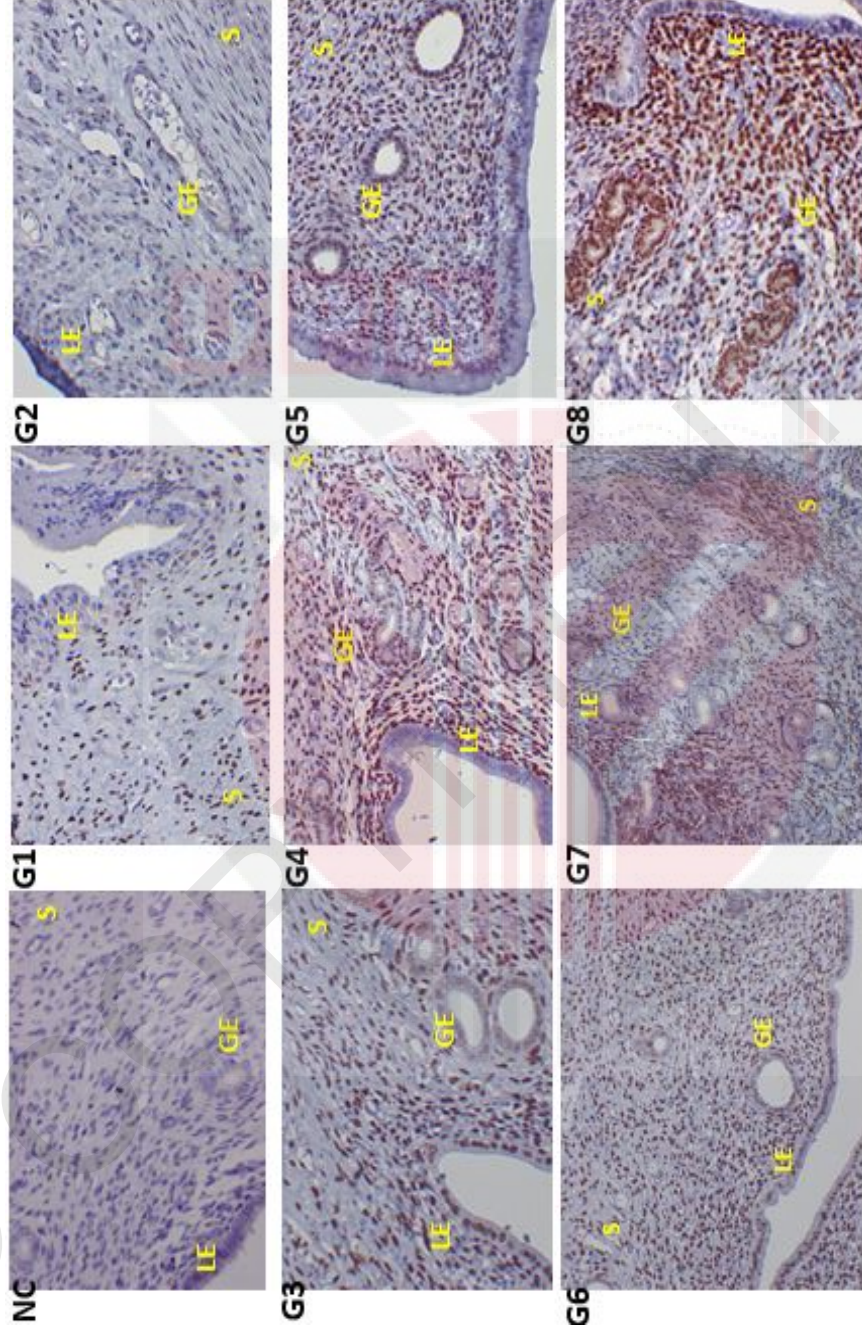


Figure 7.8: Photomicrograph of the uteri of rats of different experimental groups treated with PbA and different doses of R and HEBN showing P4R expression

*NC: without primary antibodies. G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). PR immunoreactivity is evident in GE and stroma cells, staining intensity was higher in G5 as and G8 compared to control and R and HEBN treated groups. Magnification 200x.

Table 7.5: Expression of steroid hormone receptors in uterine tissues of rats treated with different doses of REBN & HEBN exposed to PbA toxicity

Parameters	Groups							
	G1	G2	G3	G4	G5	G6	G7	G8
E2R in LE	1±0 ^c	0.5±0 ^c	1±0 ^c	2.6±0.3 ^a	3±0 ^a	1.6±0.3 ^b	3±0 ^a	3±0 ^a
E2R in GE	1.16±1.16 ^c	0±0 ^c	1±0 ^c	2.8±0.16 ^a	3±0 ^a	2±0 ^a	3±0 ^a	3±0 ^a
E2R in S	0.6±0.16 ^c	0±0 ^c	2±0 ^a	2.5±0 ^a	2.8±0.16 ^a	2±0 ^a	2±0 ^a	2.5±0 ^a
P4R in LE	0.3±0.16 ^c	0±0 ^c	2±0 ^a	0±0 ^c	0±0 ^c	2.5±0 ^a	0±0 ^a	0±0 ^a
P4R in GE	1.3±0.3 ^b	0±0 ^c	1±0 ^c	1±0 ^c	0±0 ^c	2±0 ^a	1±0 ^c	2.8±0.16 ^a
P4R in S	2±0 ^a	0±0 ^c	1±0 ^c	3±0 ^a	3±0 ^a	0.3±0.3 ^c	2.5±0 ^a	3±0 ^a

Data are expressed as Mean±SEM. Different letters a, b, c denotes significant difference at p<0.05. *G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) G4= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt), LE = uterine luminal epithelium; GE = uterine glandular epithelium; S = stromal cells.

7.3.5 Assessment of Metallothionein MT in uterine tissues

On the other hand, it was observed that MT reaction was observed significantly ($p<0.05$) higher in positive control group followed by lowest R and H EBN treated groups at a dose of (REBN at 60mg/kg bwt and HEBN at 40 mg/kg bwt), when compared to the negative control and other R and H EBN treated groups, which involved in all areas (LE, GE and stroma cells). However, significantly ($p<0.05$) low MT expressions were observed in medium to highest and REBN and highest dose of HEBN treated groups at a dose of (90 and 120 mg/kg bwt of REBN) and (60mg/kg bwt of HEBN), while a significantly ($p<0.05$) higher expression was observed in PbA treated group (G2) when compared to negative control, R and H EBN treated groups, as shown in (Figure 7.8), indicated in Table-7.4.

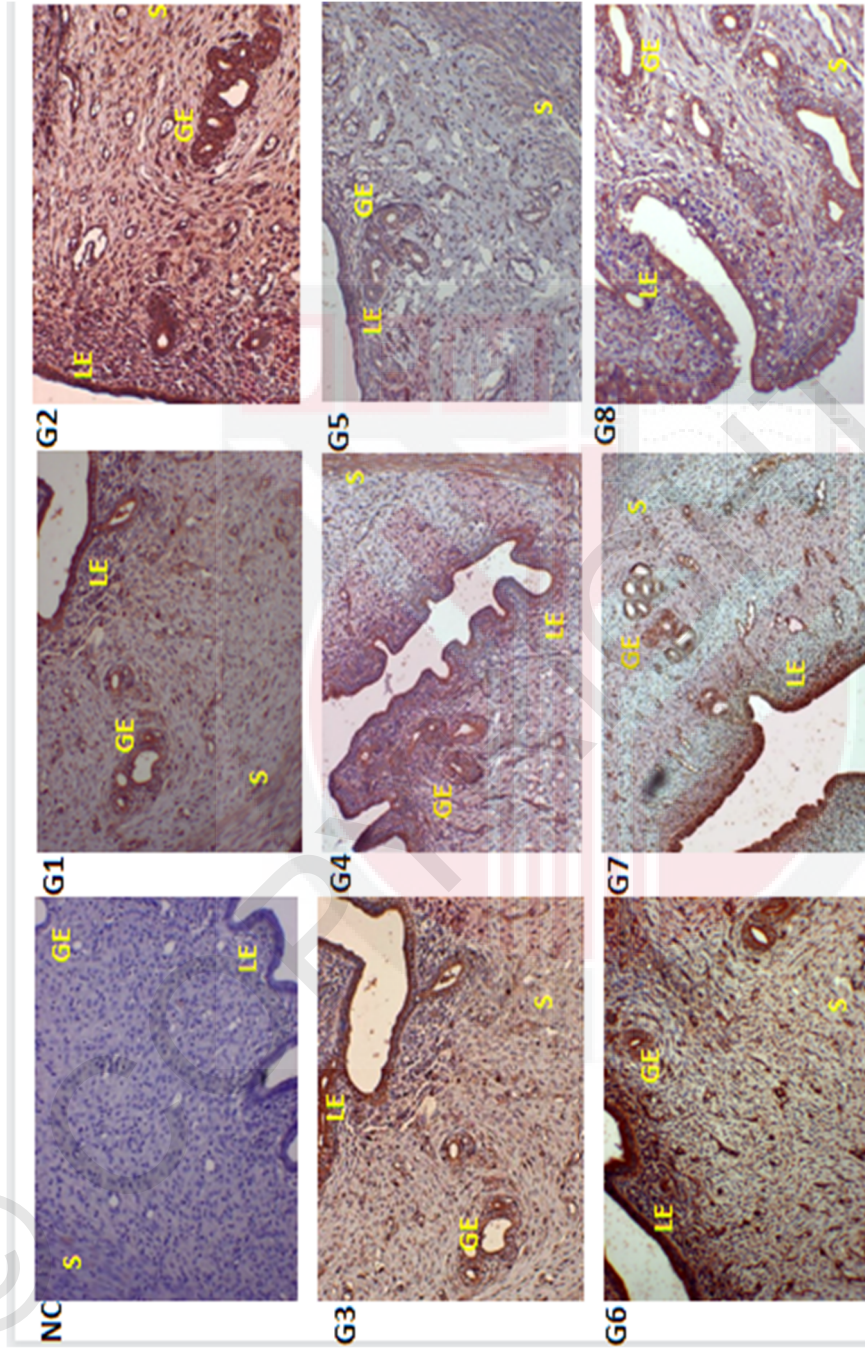


Figure 7.9: Photomicrograph of uterine tissues different experimental groups treated with PbA and different doses of R and HEBN showing MT expression

*NC: without primary antibodies. G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). MT immunoreactivity is evident in LE, staining intensity was higher in G2, G3 and G7 when compared to control and R and HEBN treated groups. Magnification 200x.

Table 7.6: Expression of MT in uterine tissues of rats treated with different doses of REBN & HEBN exposed to PbA toxicity

Parameters	Groups							
	G1	G2	G3	G4	G5	G6	G7	G8
MT in LE	0.5±0.16 ^c	3±0 ^a	1.3±0.3 ^b	1±0 ^c	0.3±0.16 ^c	2±0 ^a	1±0 ^c	0.6±0.16 ^c
MT in GE	1±0 ^c	3±0 ^a	0.5±0.16	0.5±0.16 ^c	0.16±0.16 ^c	1±0 ^c	0.5±0.16 ^c	0.16±0.16 ^c
MT in S	0.3±0.3 ^c	3±0 ^a	1.3±0.3 ^b	1±0 ^c	0±0 ^c	1±0 ^c	0±0 ^c	0±0 ^c

Data are expressed as means ± standard error mean (M±SEM). Different letters a, b, c denotes significant difference at p<0.05. *G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) G4= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). LE = uterine luminal epithelium; GE = uterine glandular epithelium; S = stromal cells.

7.3.6 Assessment and scoring of EGF and EGFR in the ovarian tissues

Descriptive immunohistochemical photomicrographs demonstrating the expressions of EGF and EGFR in the ovarian tissue sections of all the experimental groups are depicted in Figures. 7.9- 7.10 respectively, while the score variations are listed in Table-7.5. The expressions of EGF were observed significantly ($p < 0.05$) higher in ovarian surface epithelium and interstitial cells, than those noticed in highest treated groups as, G5 (REBN of 120 mg/kg bwt) and G8 (HEBN of 60 mg/kg bwt) groups (Figure-7.9), while no to very less staining for EGF expression was observed in untreated control and positive controls and other R and H EBN treated groups except for minimal staining in the ovarian surface epithelium and interstitial cells. Furthermore, EGFR showed a dense and greater quantity of interstitial cells in G4 and G5 (REBN of 90 and 120 mg/kg bwt) and G8 (HEBN of 60 mg/kg bwt) groups when compared to G2 (positive control) and rest of the R and H EBN treated groups (Figure 7.10).

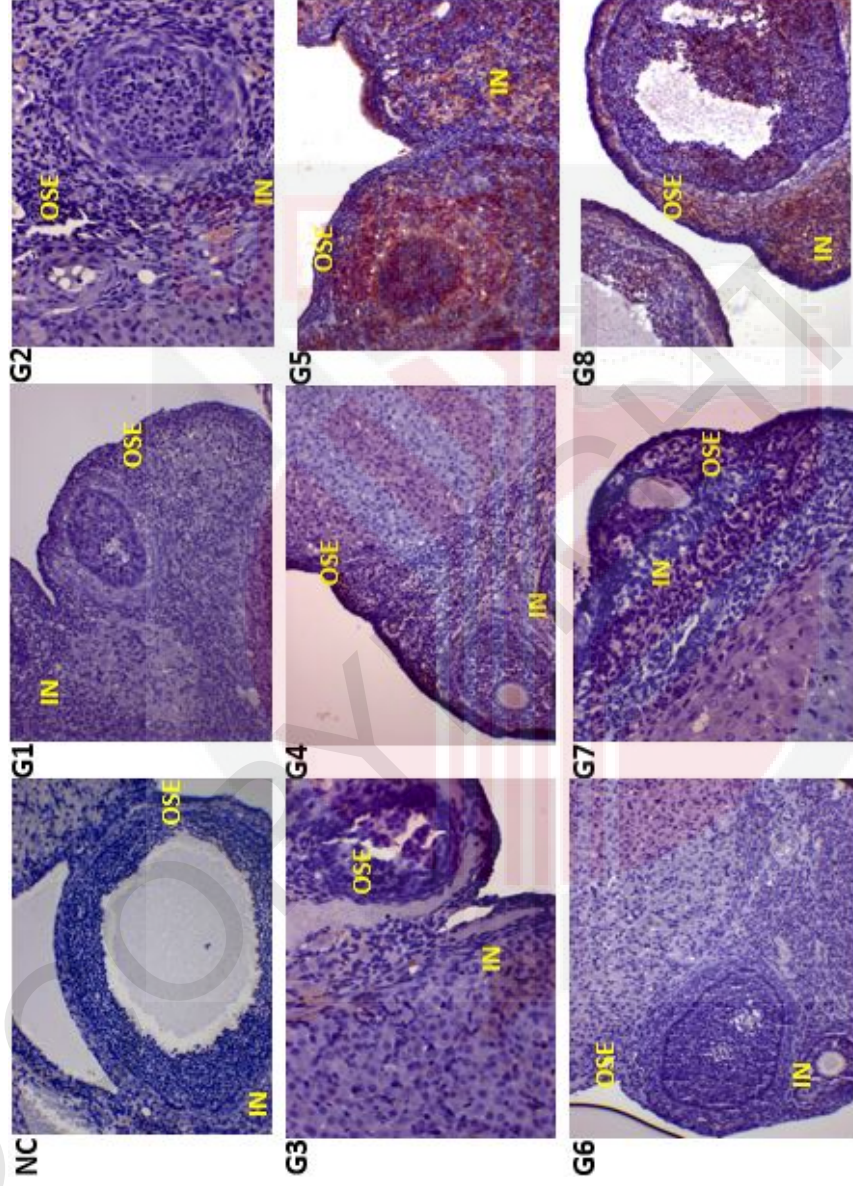


Figure 7.10: Photomicrograph of ovarian tissues different experimental groups treated with PbA and different doses of R and HEBN showing EGF expression

*NC: without primary antibodies. G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). EGF immunoreactivity is evident ovarian surface epithelium (OSE) and interstitial cells (IN), staining intensity was higher in G5 when compared to control and R and HEBN treated groups. Magnification 200x.

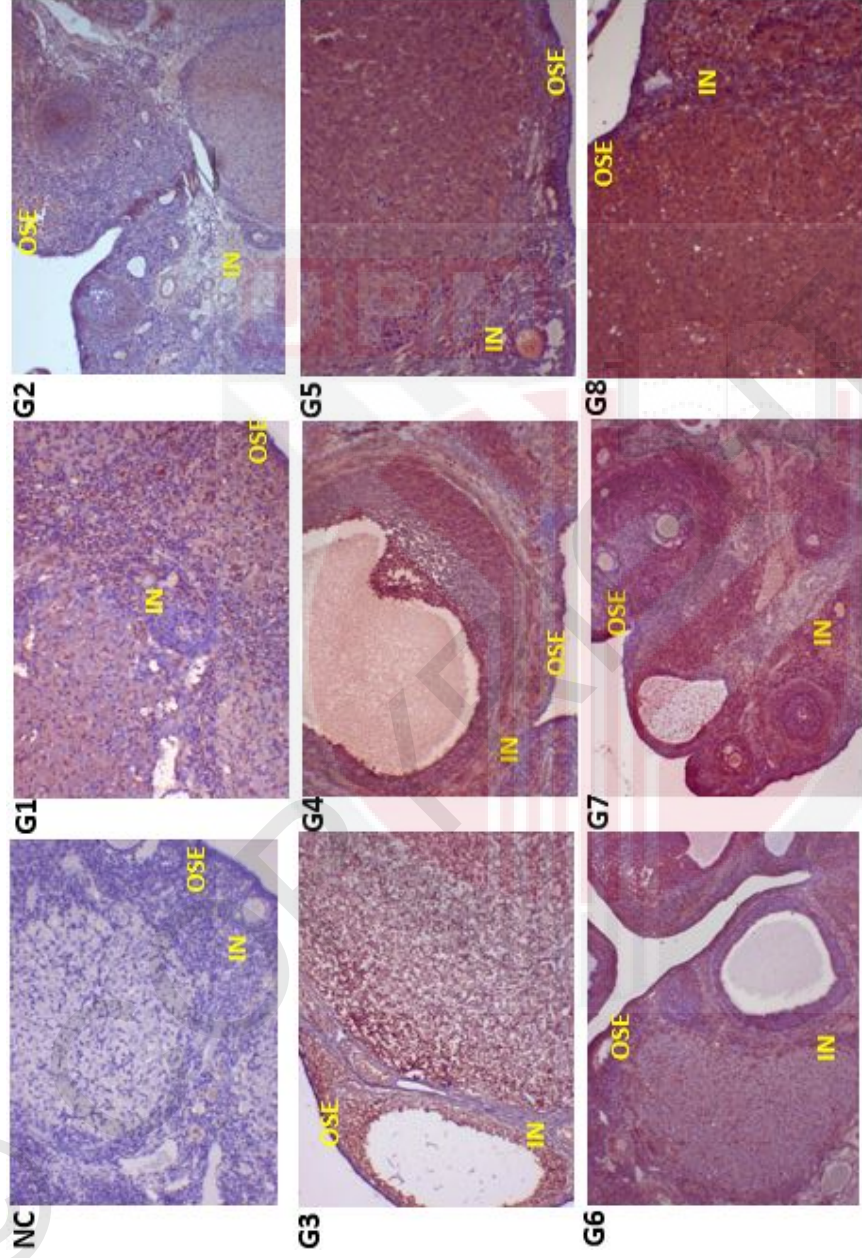


Figure 7.11: Photomicrograph of ovarian tissues different experimental groups treated with PbA and different doses of R and HEBN showing EGFR expression

*NC: without primary antibodies. G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). EGFR immunoreactivity is evident ovarian surface epithelium (OSE) and interstitial cells (IN), staining intensity was higher in G4, G5 and G8 when compared to control and R and HEBN treated groups. Magnification 200x.

Table 7.7: Expressions of EGF and EGFR in the ovarian tissues of rats treated with different doses of REBN & HEBN exposed to PbA toxicity

Parameters	Groups							
	G1	G2	G3	G4	G5	G6	G7	G8
EGF in OSE	1±0 ^c	0±0 ^c	1.3±0.3 ^c	1.6±0.8 ^b	2.8±0.16 ^a	0±0 ^c	1±0 ^c	2±0 ^b
EGF in interstitial cells	1±0 ^c	0±0 ^c	1±0 ^c	2.3±0.6 ^a	2.5±0 ^a	1±0 ^c	0±0 ^c	3±0 ^a
EGFR in OSE	1.6±0.3 ^b	0.3±0.3 ^c	0±0 ^c	2.3±0.6 ^a	2.6±0.16 ^a	2±0 ^b	2.5±0.28 ^a	3±0 ^a
EGFR in interstitial cells	1.3±0.16 ^b	0±0 ^c	1±0 ^c	2.1±0.16 ^a	3±0 ^a	2±0 ^b	2.3±0.6 ^a	3±0 ^a

Data are expressed as Mean±SEM. Different letters a, b, c denotes significant difference at p<0.05. *G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) G4= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). OSE = ovarian surface epithelium.

7.3.7 Assessment and scoring of PCNA and VEGF in the ovarian tissues

IHC expressions of PCNA and VEGF also showed a dense and greater quantity of interstitial cells in G4 and G5 (REBN of 90 and 120 mg/kg bwt) and G8 (HEBN of 60 mg/kg bwt) groups when compared to G2 (positive control) and rest of the R and H EBN treated groups (Figure 7.11- 7.12), while the score variations are listed in Table- 7.7. Though, EGFR and PCNA reactions started to be seen at 90 mg/kg bwt of REBN supplement (G4) with a similar degree of expression and staining compared with dose of EBN supplemented group G5 ($p > 0.05$) and G8 (HEBN of 60 mg/kg bwt) groups showed the significantly ($P < 0.05$) higher expression (Figure 7.11- 6.12). However, G1, G2, G3, G6 and G7 of VEGF (Figure 7.11) appeared to show some very weak reactions in positive, negative controls and low dosed groups of R and H EBN. But the highest expression was noticed in higher dose groups as (90 and 120 mg/kg bwt of REBN and HEBN of 60 mg/kg bwt) groups. Therefore, immunohistochemistry expressions in ovarian surface epithelium and interstitial cells showed significantly higher ($P < 0.05$) expressions of steroid hormones VEGF, and PCNA in medium, highest dose groups of REBN and highest dose group of HEBN when compared to rest of the R and H EBN treated, negative and positive control groups.

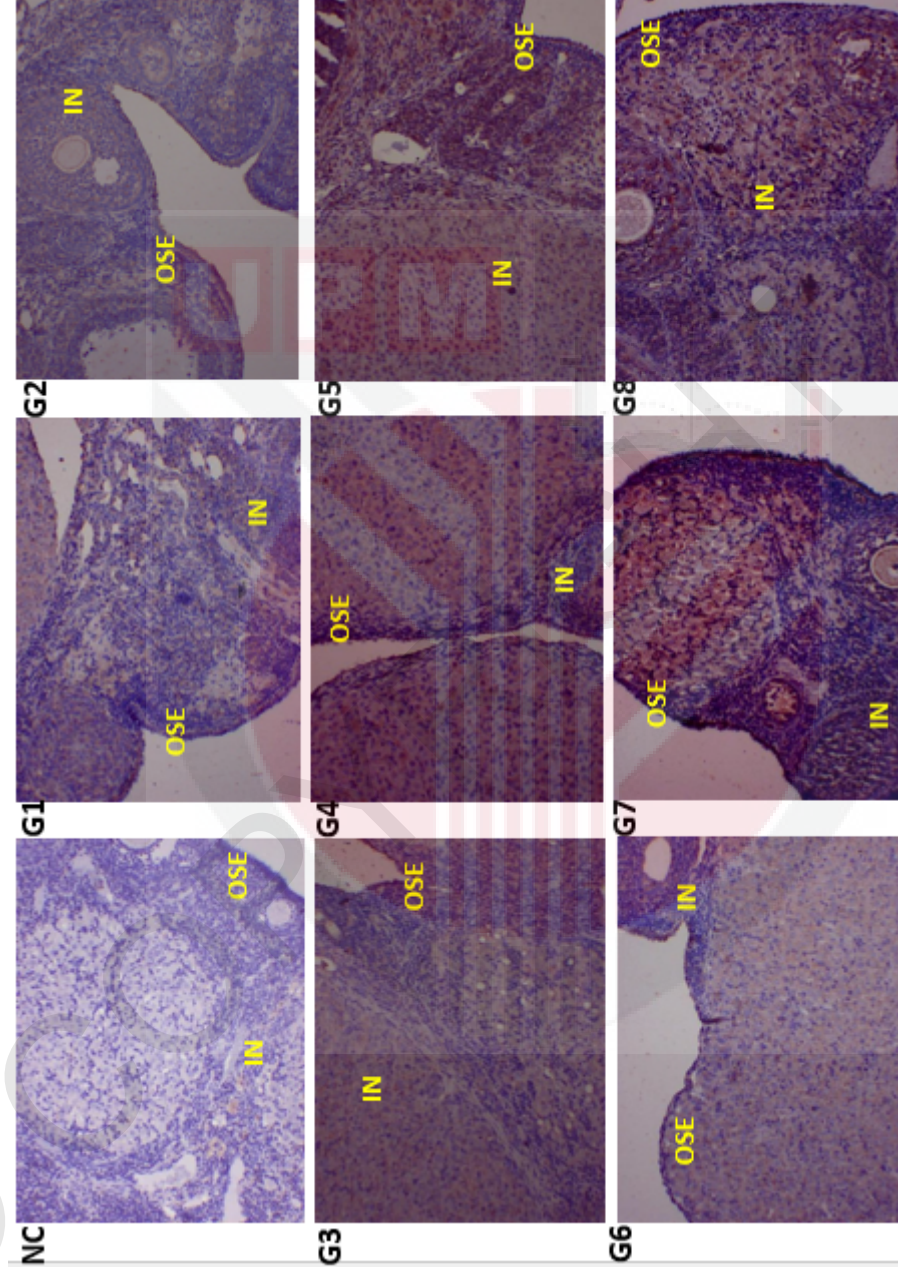


Figure 7.12: Photomicrograph of ovarian tissues different experimental groups treated with PbA and different doses of R and H EBN showing VEGF expression

*NC: without primary antibodies. G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). VEGF immunoreactivity is evident ovarian surface epithelium (OSE) and interstitial cells (IN), staining intensity was higher in G4, G5, G7 and G8 when compared to control and R and HEBN treated groups. Magnification 200x.

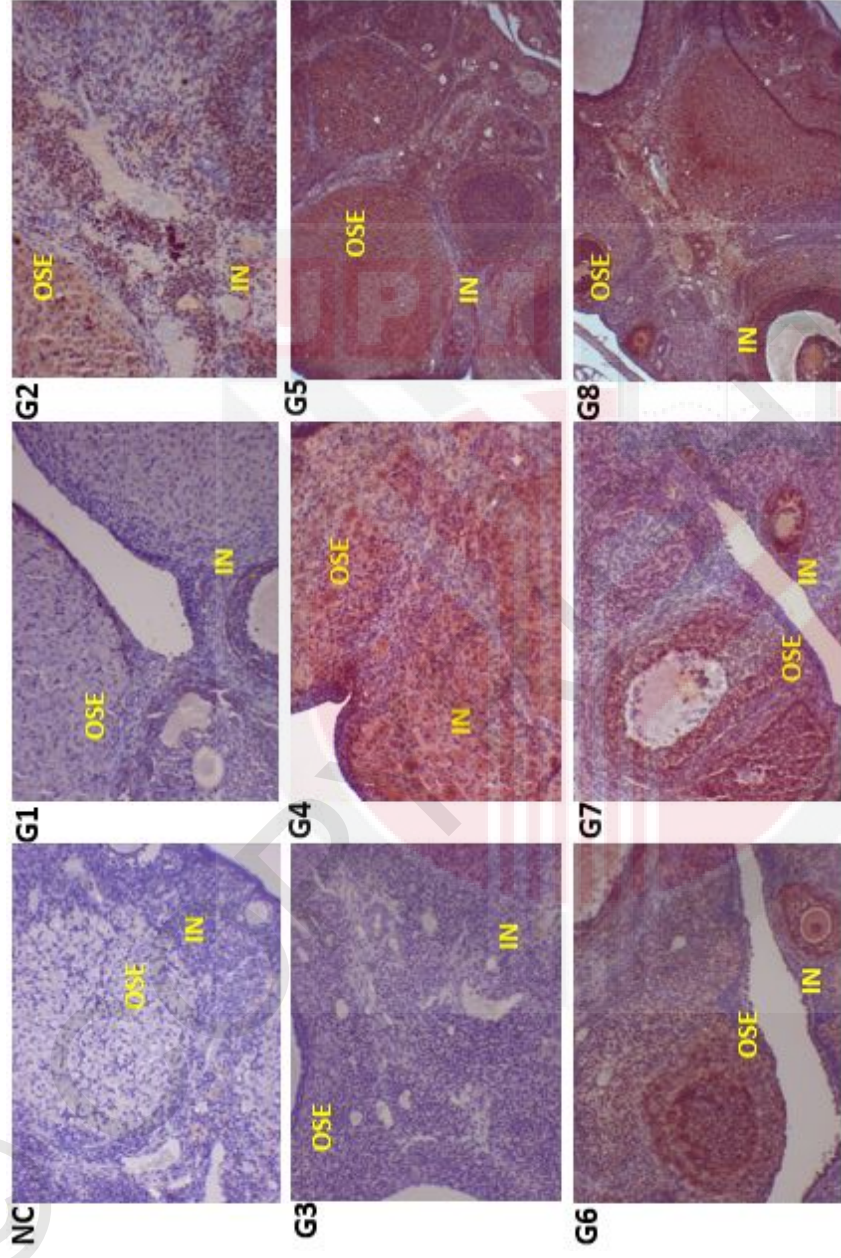


Figure 7.13: Photomicrographs of ovarian tissues of different experimental groups treated with PbA and different doses of R and H EBN showing PCNA expression

*NC: without primary antibodies. G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). PCNA immunoreactivity is evident in ovarian surface epithelium (OSE) and interstitial cells (IN), staining intensity was higher in G4, G5 and G8 when compared to control and R and HEBN treated groups. Magnification 200x.

Table 7.8: Expressions of VEGF and PCNA in the ovarian tissues of rats treated with different doses of REBN & HEBN exposed to PbA toxicity

Parameters	Groups							
	G1	G2	G3	G4	G5	G6	G7	G8
VEGF in OSE	1.3±0.3 ^b	0.3±0.16 ^c	1±0 ^c	1.8±0.16 ^b	3±0 ^a	1.6±0.3 ^b	2.3±0.3 ^a	2.8±0.16 ^a
VEGF in interstitial cells	2.5±0.28 ^a	0.3±0 ^c	2±0 ^b	1.6±0.8 ^b	2.8±0.16 ^a	1±0 ^c	2.1±0.16 ^a	3±0 ^a
PCNA in OSE	1±0 ^c	0.6±0.3 ^c	1±0 ^c	2.3±0.3 ^a	2.8±0.16 ^a	1.6±0.8 ^b	2±0 ^b	2.5±0.28 ^a
PCNA in interstitial cells	2±0 ^b	0±0 ^c	1±0 ^c	3±0 ^a	3±0 ^a	2±0 ^b	2.1±0.16 ^a	3±0 ^a

Data are expressed as Mean±SEM. Different letters a, b, c denotes significant difference at p<0.05. *G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) G4= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). OSE = ovarian surface epithelium.

7.3.8 Expression of steroid hormone receptors E2R and P4R in ovarian tissues

Representative immunohistochemical photomicrographs showing the expressions of E2R and P4R in the histological uterine sections of all the experimental groups are depicted in Figures. 7.13 and 7.14 respectively, whereas the respective score differences are listed in Table-7.8. The interstitial cell density and E2R expression in ovary increased with the increase in REBN dose. The rats treated with medium and highest REBN dose of (90 and 120 mg/kg bwt) and highest HEBN treated dose of (60mg/kg bwt) showed a significant ($p < 0.05$) increase in E2R expression compared with the negative and positive control and the other R and H EBN treated groups (Figure 6.13). On the other hand, P4R immunoreactivity significantly ($p < 0.05$) higher expression appeared only in G5 (REBN of 120 mg/kg bwt), while the lowest expressions were observed in negative and positive control and other R and H EBN treated groups (Figure 7.14).

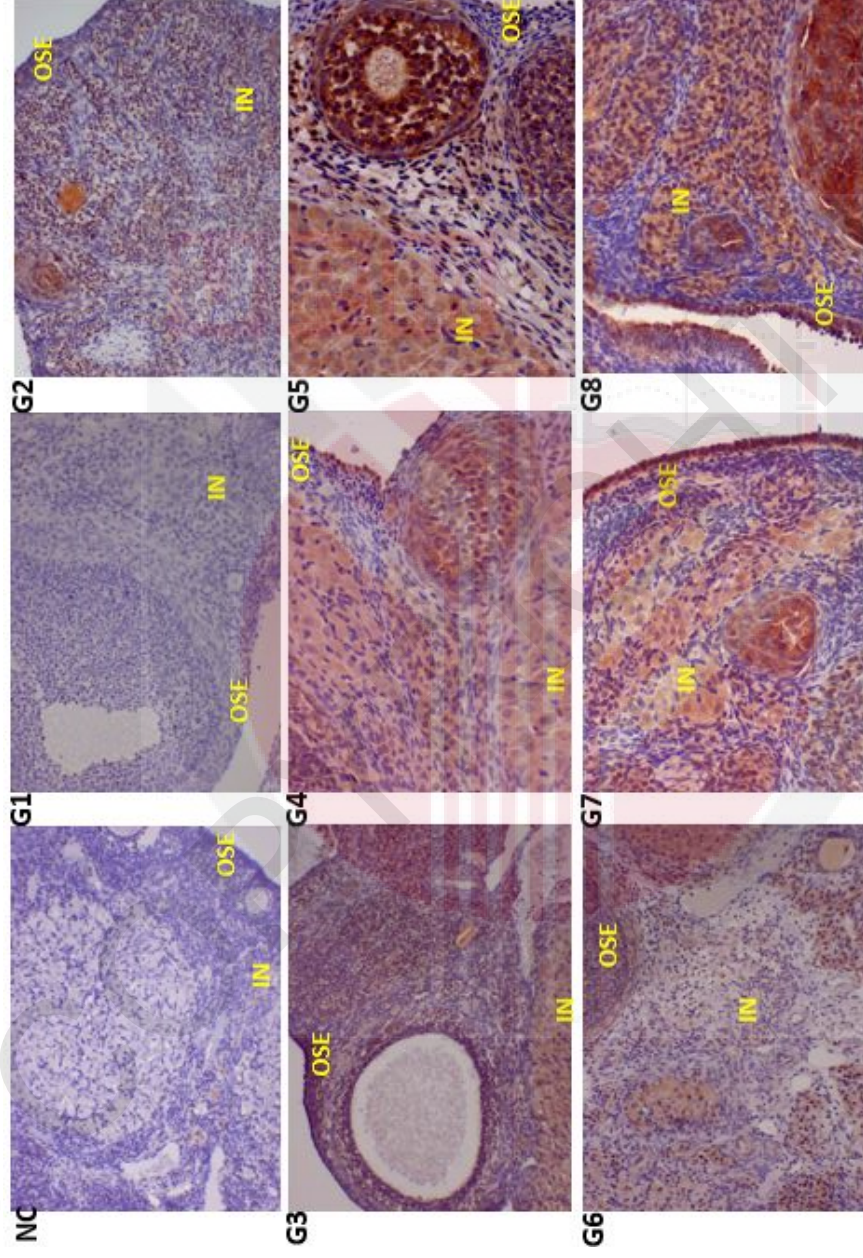


Figure 7.14: Photomicrograph of ovarian tissues different experimental groups treated with PbA and different doses of R and HEBN showing E2R expression

*NC: without primary antibodies. G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). ER immunoreactivity is evident ovarian surface epithelium (OSE) and interstitial cells (IN), staining intensity was higher in G5 and G8 when compared to control and R and HEBN treated groups. Magnification 200x.

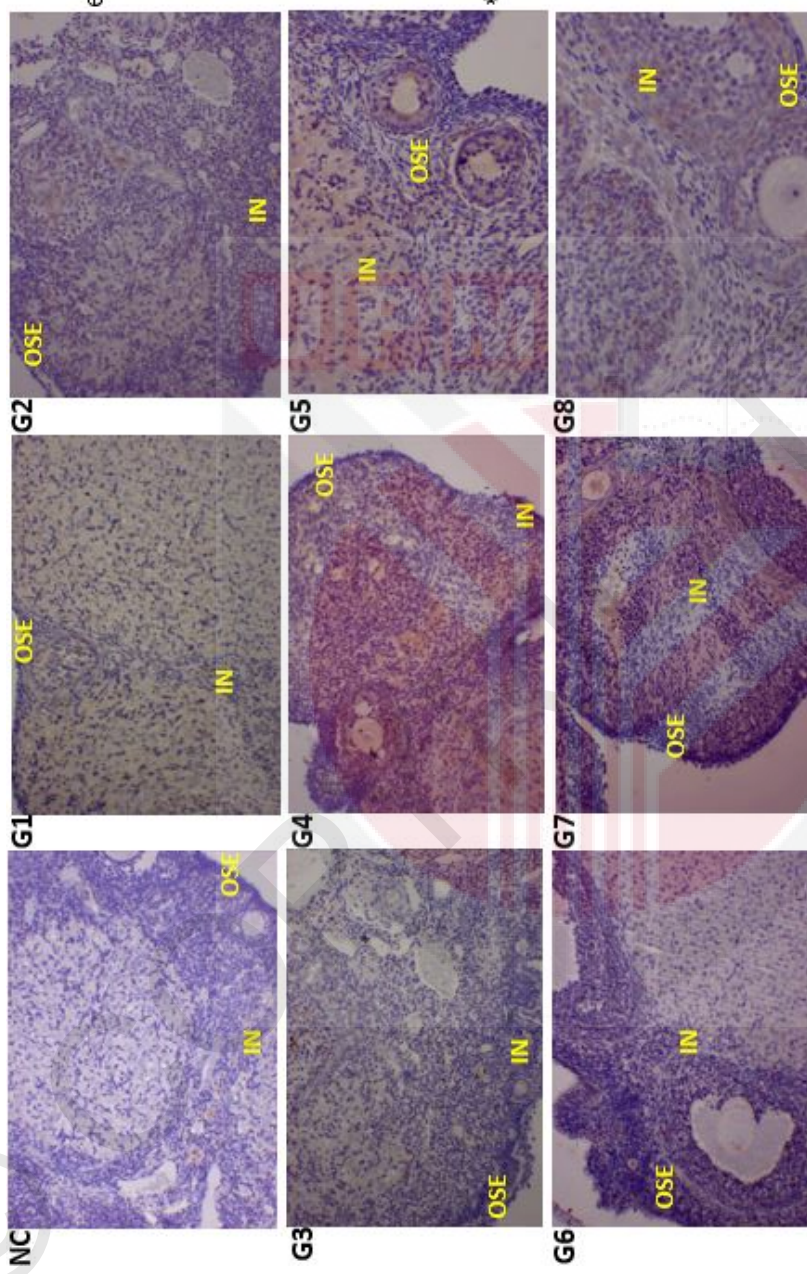


Figure 7.15: Photomicrograph of ovarian tissues different experimental groups treated with PbA and different doses of R and HEBN showing P4R expression

*NC: without primary antibodies. G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). PR immunoreactivity is evident OSE and interstitial cells, staining intensity was higher in G5 when compared to control and R and HEBN treated groups. Magnification 200x.

Table 7.9: Expression of steroid hormone receptors E2R and P4R in ovarian tissues of rats treated with different doses of REBN & HEBN exposed to PbA toxicity

Parameters	Groups							
	G1	G2	G3	G4	G5	G6	G7	G8
E2R in OSE	1±0 ^c	0.5±0 ^c	1±0 ^c	2.6±0.3 ^a	2.8±0.16 ^a	0.3±0.16 ^c	3±0 ^a	3±0 ^a
E2R in interstitial cells	1.16±0.16 ^b	1±0 ^c	1.3±0.3 ^b	2.8±0.3 ^a	3±0 ^a	1.3±0.3 ^b	2.5±0.2 ^a	2.8±0.16 ^a
P4R in OSE	0.3±0.16 ^c	0±0 ^c	0±0 ^c	1±0 ^c	3±0 ^a	1±0 ^c	1±0 ^c	2.5±0 ^a
P4R in interstitial cells	1.3±0.3 ^b	0±0 ^c	1±0 ^c	2.8±0.16 ^a	2.5±0.2 ^a	1±0 ^c	2.5±0.2 ^a	2.6±0.3 ^a

Data are expressed as Mean±SEM. Different letters a, b, c denotes significant difference at p<0.05. *G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) G4= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). OSE = ovarian surface epithelium

7.3.9 Expression of Metallothionein MT in ovarian tissues

A significantly ($p < 0.05$) increased immunohistochemical expressions of MT in ovarian tissues which were assessed by the number of positive stained cells were observed to be significantly higher ($P < 0.05$) in positive control (PbA of 10 mg/kg bwt), when compared to the negative control and other treated R and H EBN groups, while the lowest staining was observed in the highest EBN treated with G5 (REBN of 120 mg/kg bwt) and G8 (HEBN of 60 mg/kg bwt) dose groups (Figure 7.15), indicated in Table: 7.9.

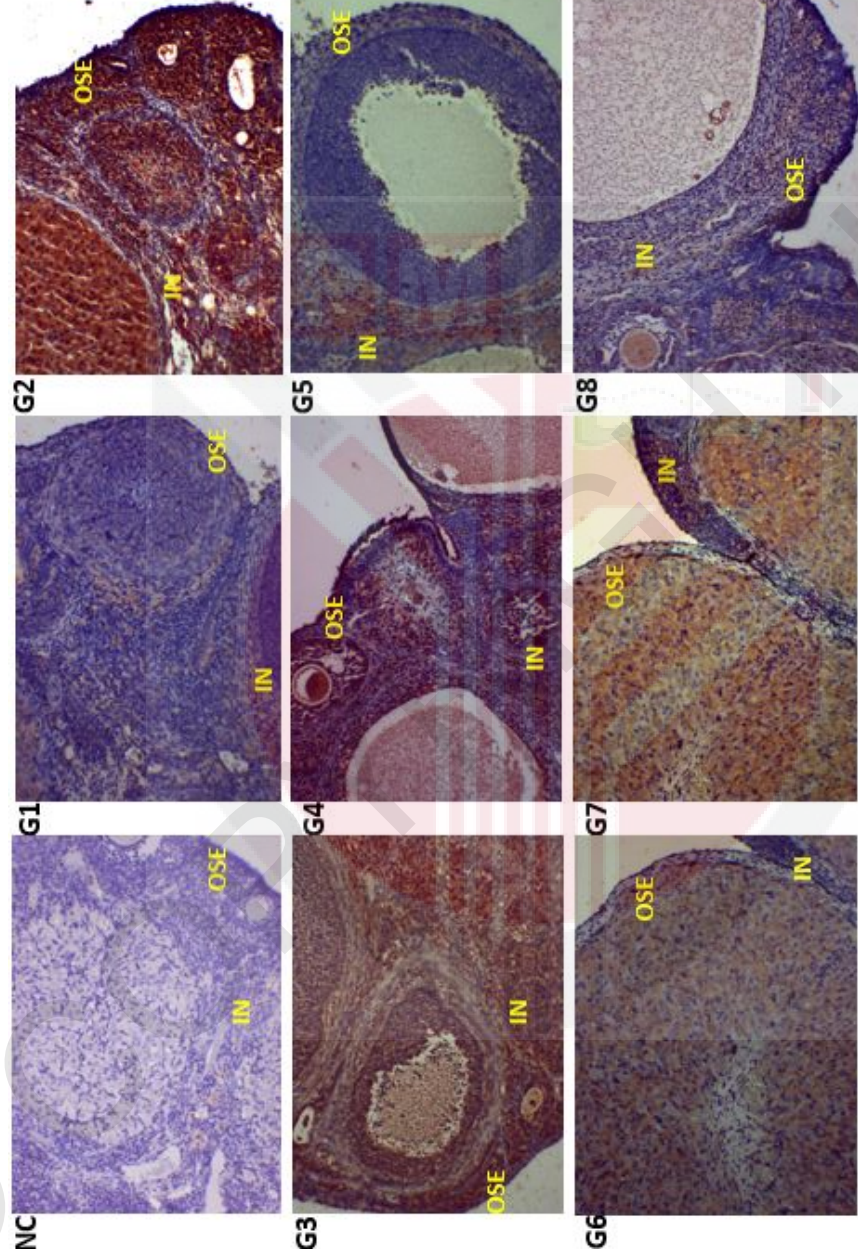


Figure 7.16: Photomicrograph of ovarian tissues different experimental groups treated with PbA and different doses of R and HEBN showing MT expression
 *NC: without primary antibodies. G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). MT immunoreactivity is evident OSE and interstitial cells, staining intensity was higher in G2 when compared to control and R and HEBN treated groups. Magnification 200x.

Table 7.10: Expression of steroid hormone receptors and MT in ovarian tissues of rats treated with different doses of REBN & HEBN exposed to PbA toxicity

Parameters	Groups							
	G1	G2	G3	G4	G5	G6	G7	G8
MT in OSE	0±0°	3±0 ^a	3±0 ^a	0±0°	0±0°	0±0°	0±0°	0±0°
MT in interstitial cells	0.3±0.16°	3±0 ^a	3±0 ^a	1±0°	0.5±0°	1.3±0.3°	1±0°	0.3±0.16°

Data are expressed as Mean±SEM. Different letters a, b, c denotes significant difference at p<0.05. *G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) G4= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). OSE = ovarian surface epithelium

7.3.10 Immunohistochemical expressions of MT in kidney and liver tissues

In the kidney tissue samples examined expressions of MT were found in the renal proximal convoluted tubular cells, and few cells in glomeruli and distal convoluted tubules were observed to be significantly higher ($P < 0.05$) in the positive control group, while the lowest expression of MT was observed in negative control, medium and highest G4 and G5 (REBN of 90 and 120 mg/kg bwt) and G8 (HEBN of 60 mg/kg bwt) dose groups, when compared to lowest REBN and HEBN treated groups (Figure 7.16). Increased immunohistochemical expressions of MT in liver cells as assessed by the positive stained cells were observed to be significantly higher ($P < 0.05$) in G2 (positive control), when compared to lowest REBN and HEBN treated, and the least MT expression was observed in and G1 (untreated control), medium and highest G4 and G5 (REBN of 90 and 120 mg/kg bwt) and G8 (HEBN of 60 mg/kg bwt) dose groups (Figure 7.17). Furthermore, the mean score differences of histopathological lesions in the kidney and liver among the groups are shown in Table 7.10.

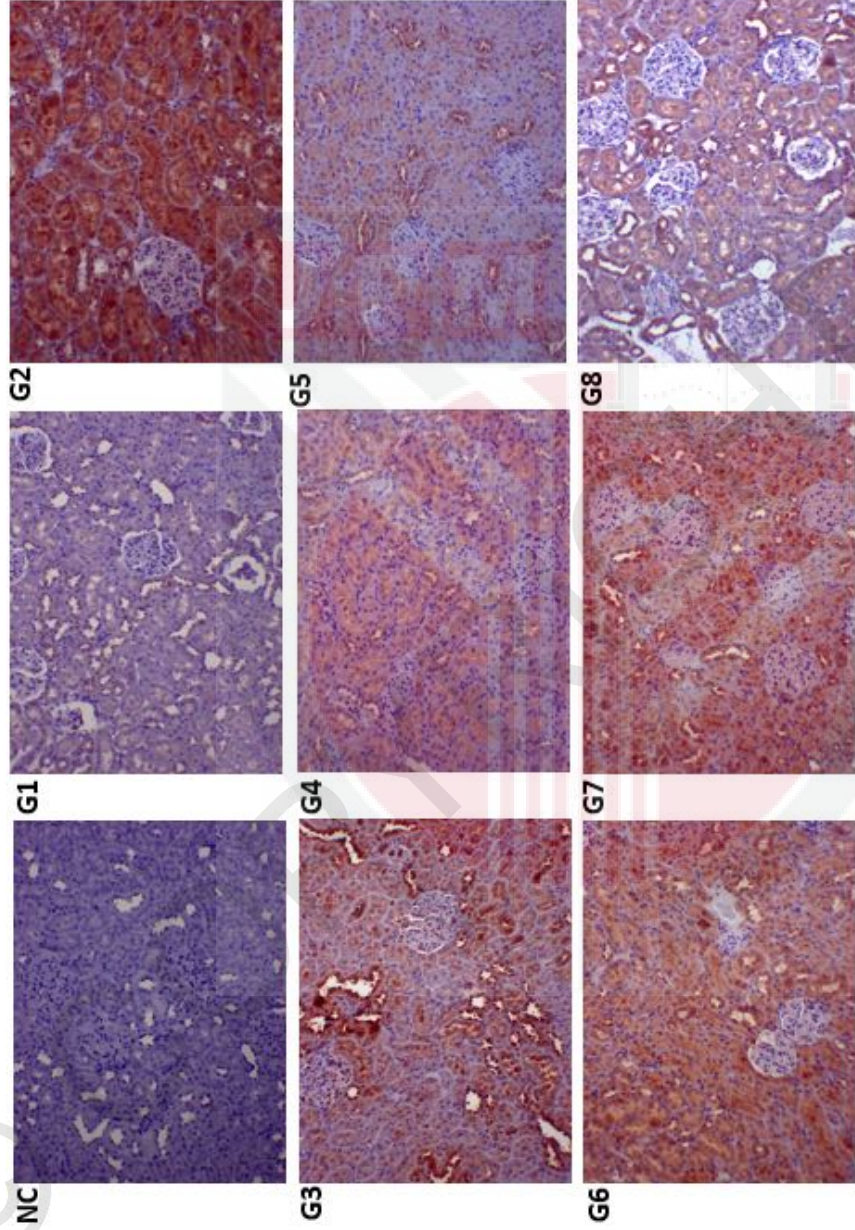


Figure 7.17: Photomicrograph of kidney tissues of different experimental groups treated with PbA and different doses of R and HEBN showing MT expression

*NC: without primary antibodies. G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). MT immunoreactivity is evident in cytoplasm and interstitial cells, staining intensity was higher in G2 as compared to negative control and R and HEBN treated groups. Magnification 200x.

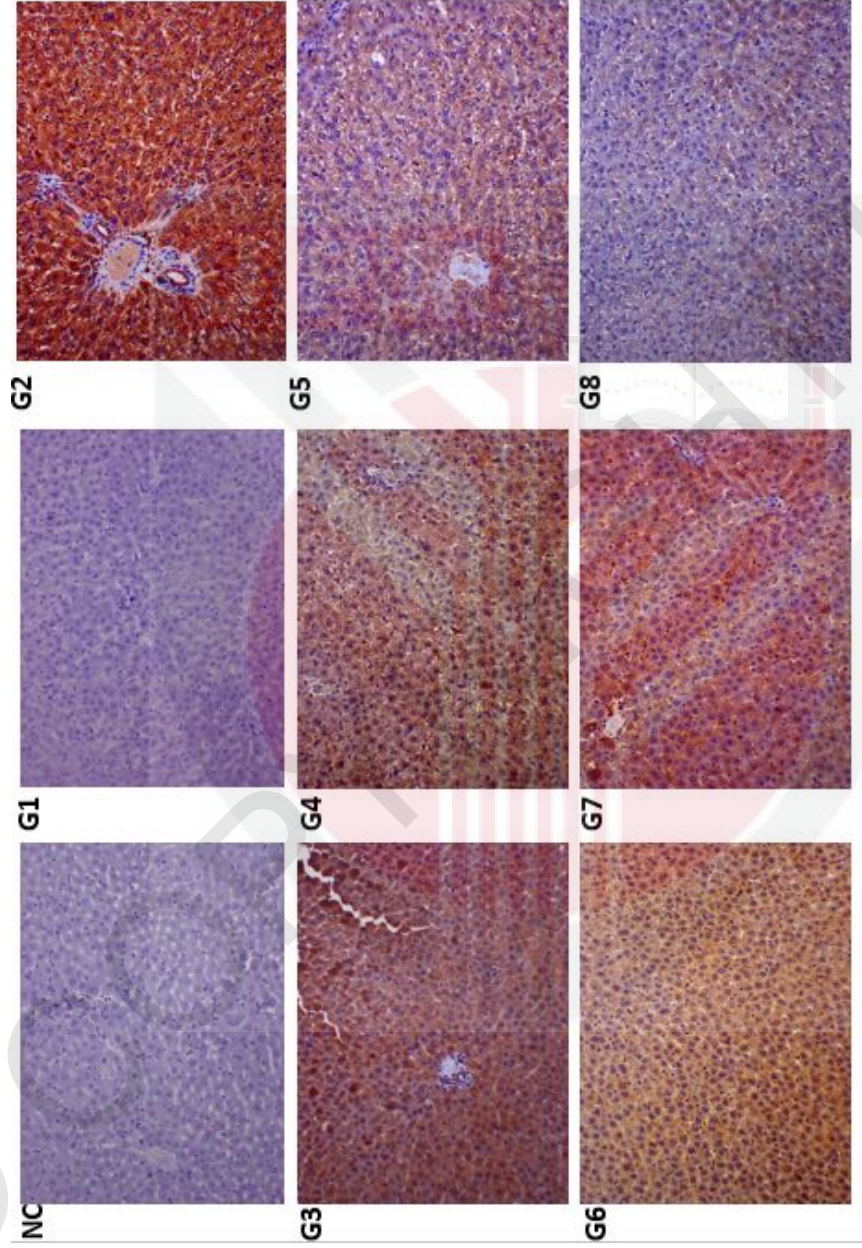


Figure 7.18: Photomicrograph sections of liver tissues of different experimental groups treated with PbA and different doses of R and HEBN showing MT expression

*NC: without primary antibodies. G1=Control, G2= PbA (10 mg/kg bwt), G3= (REBN 60 mg/kg bwt) G4= (REBN 90 mg/kg bwt) and G5= (REBN 120 mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). MT immunoreactivity is evident in cytoplasm and interstitial cells, staining intensity was higher in G2 when compared to negative control and R and HEBN treated groups. Magnification 200x.

Table 7.11: Mean scores of MT expression in kidney and liver tissues of rats supplemented with REBN and HEBN exposed to PbA toxicity

Parameters	Groups							
	G1	G2	G3	G4	G5	G6	G7	G8
MT in nuclei of kidney	0±0 ^a	2.6±0.3 ^c	2±0 ^b	0±0 ^a	0±0 ^a	0.3±0.1 ^a	0±0 ^a	0±0 ^a
Kidney MT in cytoplasm of kidney	0±0 ^a	3±0 ^c	2±0 ^b	0.6±0.1 ^a	0.1±0.1 ^a	2±0 ^b	1±0 ^a	0.1±0.1 ^a
MT in cytoplasm of liver	0±0 ^a	0±0 ^a	0±0 ^a	0±0 ^a	0±0 ^a	0±0 ^a	0±0 ^a	0±0 ^a
Liver MT in cytoplasm of liver	0±0 ^a	3±0 ^c	3±0 ^c	1±0 ^a	0.5±1 ^a	2.1±0.4 ^b	1±0 ^a	0±0 ^a

Data are expressed as Mean±SEM. Different letters a, b, c denotes significant difference at p<0.05. *G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) G4= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt). MT= Metallothionine staining of nuclei and cytoplasm in liver cells treated with different doses of EBN against PbA=10mg/kg bwt

7.3.11 The concentrations of PbA in uterine tissues measured by ICP-MS

Figure 7.18 shows the concentrations of PbA in the rat's uterine tissues of the different experimental groups. The result showed the highest expression and toxicity level in G2 (the positive control) as compared to the G1 (untreated control) and other R and H EBN treated groups. Among the experimental groups, G4, G5 (REBN 90 and 120 mg/kg bwt) and G8 (HEBN 60 mg/kg bwt) showed significantly ($p<0.05$) lowest toxicity levels compared to other R and H EBN treated groups. However, the control group (G1) showed the lowest PbA level among all the groups. ICP-MS results showed significantly ($p<0.05$) highest PbA concentration in positive control when compared to negative control group and significantly lowest concentrations were observed in medium to highest dose of REBN and highest dose HEBN.

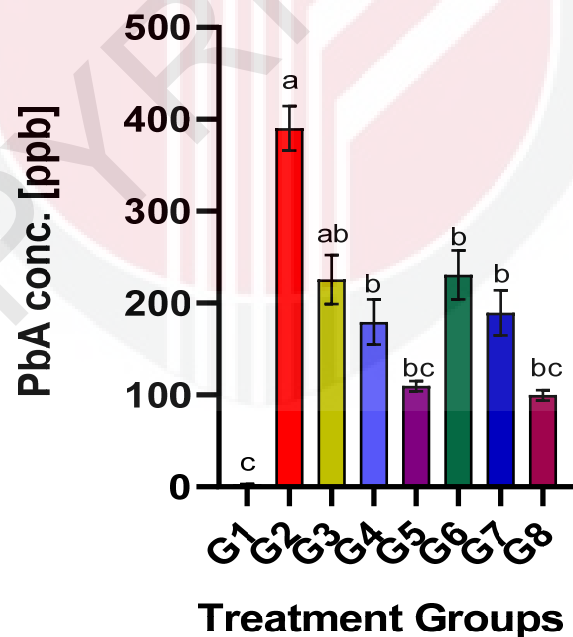


Figure 7.19: Effect of R and HEBN on concentration of PbA toxicity in the rat's uterine tissues

Data are expressed as Mean $\pm$ SEM. Different letters a and b within rows denotes significant difference at $p < 0.05$. Note: *G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) G4= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt).

7.3.12 Effect of REBN & HEBN on SOD and TBARS levels

On the other hand, SOD was lower in the positive control and observed significantly ($p<0.05$) higher in the G4, G5 (REBN 90 and 120 mg/kg bwt) and G8 (HEBN 60 mg/kg bwt) groups when compared to R and H EBN treated groups and negative control group. The changes significantly increased in the treatment groups and attained the highest value ($p<0.05$) in the REBN of 90 and 120 mg/kg bwt (G4 and G5) and HEBN of 60 mg/kg bwt (G8) groups (Figure 7.19).

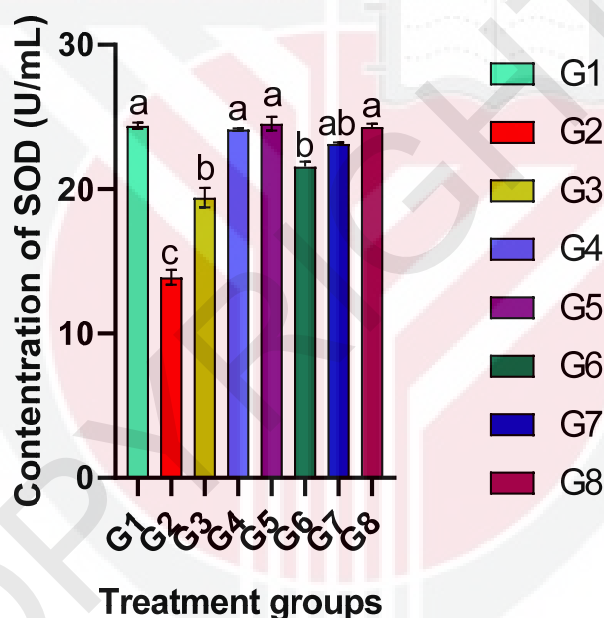


Figure 7.20: Effect of PbA administration on SOD in the R and HEBN dose groups when compared to the control and PbA group. SOD = Superoxide Dismutase

Data are expressed as Mean±SEM. Different letters a and b within rows denotes significant difference at $p < 0.05$. Note: *G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) G4= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt).

TBARS were lower in the G4, G5 (REBN 90 and 120 mg/kg bwt) and G8 (HEBN 60 mg/kg bwt) groups when compared to other R and H EBN treated and negative control groups. However, TBARS was observed significantly higher ($p<0.05$) in the positive

control (G2) group compared to the R and H EBN treated and negative control groups. The change in TBARS significantly decreased in the treatment groups and attained the highest value ($p<0.05$) in positive control group (Figure 7.20).

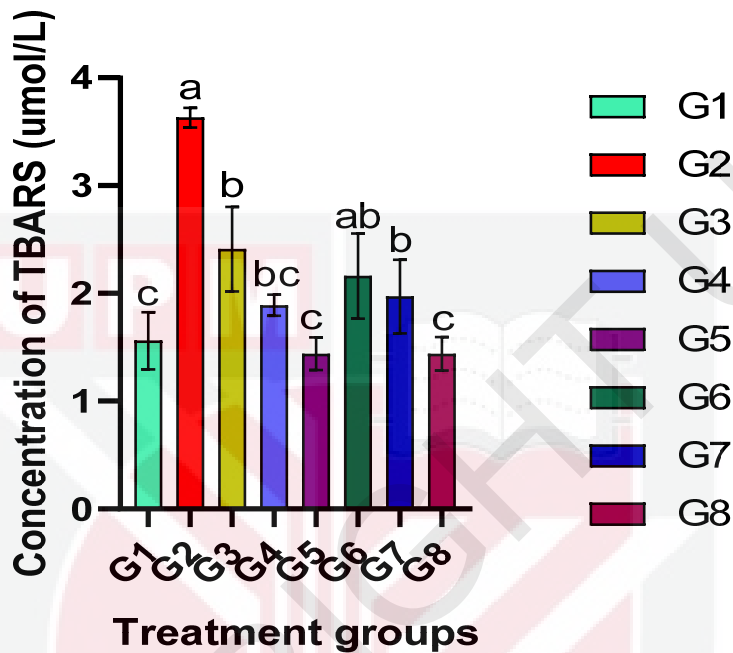


Figure 7.21: Effect of PbA administration on TBARS in R and HEBN dose groups when compared to control and PbA group TBARS =thiobarbituric acid reactive substance

Data are expressed as Mean±SEM. Different letters a and b within rows denotes a significant difference at $p < 0.05$. Note: *G1=Control, G2= PbA (10mg/kg bwt), G3= (REBN 60mg/kg bwt) G4= (REBN 90mg/kg bwt) and G5= (REBN 120mg/kg bwt), G6= (HEBN 20 mg/kg bwt); G7= (HEBN 40 mg/kg bwt); G8= (HEBN 60 mg/kg bwt).

7.4 Discussion

When orally ingested, lead can be absorbed by both humans and animals. It has been observed that a higher proportion of children (40%) and adults (10%) acquire lead through the gastrointestinal system, compared to non-ruminant and ruminant animals, where the percentages are 10% and 3%, respectively. Once absorbed, lead enters the bloodstream and primarily binds to erythrocytes, remaining in circulation before

eventually diffusing into bones and other soft tissues. This accumulation, along with the interaction between lead and blood, can reach toxic levels, posing a significant risk to health (Ali et al., 2020). Lead accumulates primarily in bone tissue after exposure (Rabinowitz et al., 1991; Satarug et al., 2002). Previous research has shown that factors including age and hormone production can trigger re-released lead to general circulation (Horiguchi et al., 2013). Therefore, lengthy half-lives are a defining feature of lead. Generally, LA has been found to have negative effects on the reproductive organs and fertility of adult female rats (Eugenia et al., 2009). The present study's vaginal histology findings demonstrated that Oestrous cycles are unaffected by PbA exposure, along with EBN. In addition, the previous study demonstrated that three out of four cycle features were normal for the treated monkeys prior to lead exposure. Menstruation may be affected by lead exposure if it is long-term. Longer and more irregular intercycle intervals and fewer days of vaginal bleeding were seen in lead-treated monkeys compared to controls (Laughlin et al., 1987). Loss in bwt, uterine weight, and the number of ova and conceptions in mice were all seen after treatment of PbA at varying doses (Odenbro & Kihlström, 1977). The vaginal cytological investigation demonstrated a normal estrous cycle regardless of the subjects' exposure to PbA in the current study. A recent study reported that EBN supplementation had a positive effect on the uterus of normal rats (Albishtue et al., 2019). Exposure to PbA affects the female reproductive system by impairing menstruation, reducing fertility potential, altering hormonal production, affecting blood circulation, and influencing pregnancy and its outcome. Prior research determined that the cycles of treated primates were normal prior to lead exposure and that three of the four cycle characteristics were normal during the initial lead exposures. In contrast, chronic exposure may cause menstrual irregularities. Comparing the Pb-exposed group to the

control group, the Pb-exposed group had more variable intercycle intervals and fewer days of vaginal bleeding (Strauss et al., 2019). Bone Pb may be a significant source of target organ exposure under certain conditions, such as pregnancy, kidney disease, and menopause. Lead exposure may have a detrimental impression on uterine physiological functions such as enzymes, cytokines, growth factors, and hormones. EBN may play a role of prophylactic regimen, reproduction, and fertility, as well as in the maintenance of health (Filant et al., 2014).

EBN is known to enhance expressions of growth factors including interleukine-6, VEGF, EGF, EGFR and PCNA (Albishtue, et al., 2019) . The impression of EGF-like activity found in EBN has also been reported to associate with the important role in cell division, growth and tissue regeneration enhancement (Ma et al., 2012). These prior reports are in correspondence with the present study, which confirmed the significant expression of EGF associated with abundant proliferation of ovarian surface epithelial and interstitial cells. Furthermore, an EBN possesses the ability to potentiate mitogenic responses and provide viability, as it directly effects the proliferation of human adipose-derived stem cells, macrophage cell lines, and human colonic adenocarcinoma cell line (Caco-2) cells. Also, a lower dose of EBN could be used to synergistically induce cell proliferation and functional maintenance, especially for healing corneal wounds. The enhanced cell proliferation observed in response to EBN is due to the presence of EGF (Kong et al., 1987). Hence, the properties of EBN, like cell proliferation and the ability to potentiate mitogenic responses, can be considered effective in promoting the healing process in corneal wounds (Abidin et al., 2011). By blocking caspase-3 activation, the apoptotic cells in embryos can be suppressed thanks to EBN's vascular endothelial growth factor (VEGF) and

interleukin-6 (IL-6) content (Jin et al., 2001). Testosterone, E2, P4, luteinizing hormone (LH), follicle stimulating hormone (FSH), and prolactin have all been found in EBN, according to a previous analysis of its chemical make-up (F. Ma & D. Liu, 2012a). VEGF, FSH, and estradiol have all been shown alone and in combination to have anti-apoptotic, anti-caspase 3 activating, and proliferative effects (Bussemann et al., 2006; Vitale et al., 2002). Some of these crucial bioactive components found in EBN may explain the effect it had on the uterus in the current investigation. EBN contains protein, carbohydrates, ash, and lipids (in that order) as its nutritional components (Ma & Liu, 2012a). Water-soluble components of EBN, including proteins, carbohydrates, iron, inorganic salts, and fiber, contribute to the food's high respect as a dietary supplement (Ma & Liu, 2012b). Similar to the EBN it mimics, sialic acid contains chemical processes that lead to cell growth. Previous research has shown that the hormone estradiol controls the production of a sialic acid binding protein in the human endometrium (Sen et al., 2001). There is evidence that the EGF-like component in EBN promotes cell proliferation, growth, and tissue regeneration. This mechanism may be one explanation for why EBN has restorative effects (Y. Kong et al., 1098).

Higher EBN dosages boosted uterine cell proliferation in rats exposed to PbA. EBN boosted uterine tissue cell proliferation in rats by increasing PCNA, a marker for DNA synthesis and cell proliferation. These findings supported EBN's significance in reproduction and fertility (Quddus et al., 2021). EBN contains VEGF and Interleukin-6 (IL-6), which prevent embryonic neuron apoptosis. Also, IL-6 inhibits the activity of caspase-3, resulting in the suppression of apoptotic cell death. (Wong et al., 2012; Alan et al., 2015). The effect of EBN on the uterus in the present study could be

attributed to some of the key bioactive compounds it contains, such as VEGF, FSH, and estradiol. (Almodovar et al., 2009). EBN was able to enhance the expression of several key growth factors and cell proliferation markers, including EGF, EGFR, VEGF, and PCNA, in the GE, LE, and uterine stromal cells. These results indicate that EBN has a proliferative effect on these cells, which may serve to mitigate the toxicity of PbA (Albishtue et al., 2019).

A prior study also reported the presence of reproductive hormones including testosterone, E₂, P₄, LH, FSH as well as prolactin in the EBN (Ma et al., 2012). Oestrogen helps the endometrium and glandular tissue expand, whereas progesterone is needed for implantation and conception. Disruptions in plasma hormone levels can cause fertility concerns and poor reproductive success by affecting ovulation, uterine lining receptivity, and reproductive system health (Albishtue et al., 2018). Oestrogen and progesterone receptors are mostly in the cell nucleus, unlike other steroid receptors. EBN dosages increase progesterone receptor (PR) expression in glandular epithelial (GE) and stromal cells. During implantation, stromal cells and GE expressed more PR. EBN may improve PR expression in the uterus, supporting its potential reproductive health benefits (Albishtue et al., 2019).

A previous study found that 60mg/kg of HEBN supplement may protect against the negative effects of PbA and promote uterine structure growth compared to the negative control group, which did not receive EBN and PbA therapy. These findings are consistent with recent studies that Pb-induced lowering of serum levels of insulin-like growth factor-1 (IGF-1), which increases uterine luminal epithelium (LE) thickness, reduces LE thickness (Diantini et al., 2018, Altmä et al., 2019, Ogunlade et al., 2020).

HEBN supplementation on uterine, glandular, and stromal cells showed a dose-dependent effect of 60 mg/kg bwt on growth factors. SEM showed pinopodes from the uterine luminal epithelium's apex during the window of receptivity in rats and humans (Quinn and Casper, 2009). Presence of Pinopodes and microvilli were indicated in the uterine luminal epithelium of the group treated with 60 mg/kg of HEBN, but not in the control group or PbA exposed. HEBN is reported to contain several growth factors, including interleukin-6, VEGF, EGF, EGFR, and PCNA, which may contribute to its positive effects on endometrial receptivity and EGF is also linked to tissue regeneration and cell division (Choy et al., 2021). HEBN enhances mitogenic responses and promotes cell proliferation in a variety of cell lines, including human adipose-derived stem cells, macrophage cell lines, and human colonic adenocarcinoma cell lines. This is due to the presence of growth factors such as EGF in HEBN stimulates cell growth and proliferation in numerous tissues (Quek et al., 2018).

It has been shown that several chelating substances, like EDTA, DTPA, and PbA, may even have a synergistic effect with Pb. According to Chisolm et al. (1963), EDTA therapy in Pb-exposed children inhibits red cell ALA dehydratase and increases urine ALA levels. Hofmann et al. (1975) reported that rats treated with the chelating agents DTPA and EDTA also experienced a decrease in ALA dehydratase activity (Hofmann et al., 1975).

Pb has a prolonged half-life and high dispersion. Pb immediately rises BLL (blood lead level) and diffuses throughout the body. Bones store 70% of lead in children. Chelating chemicals remove lead from blood and soft tissues. Lead accumulated deep in the bones can take years to migrate into the circulation and be eliminated (Schroder

et al., 2015). Chelation therapy is a recommended for the treatment and management of chronic Pb poisoning. Prevention of Pb toxicity can be terminated by chain reaction, which involves inactivation of ROS generation at molecular level. Natural phyto-antioxidant are naturally occurring polyphenolic compounds and can reduced or prevent the OS by forming a complex with lead and also by breaking the chain reaction (Terao et al., 2009). EBN is generally believed to be produced by seven species of swiftlets of the genus *Collocalia* and *Aerodramus* (Dai et al., 2021). Emerging research findings on EBN have shown that it enhances the production of reproductive hormones, such as estrogen, and serves as an antioxidant property. EBN's antioxidant properties have been reported by (Yew et al., 2014, Yida et al., 2015). It was found that the antioxidant activity of EBN treated with papain for two hours has the highest antioxidant values with a DPPH scavenging value of $29.9 \pm 4.9\%$ (Zulkifli et al., 2019). Hydrolyzed EBN is rich in protein, amino acid, sialic acid content (Fan et al., 2022). Wang et al., (2020) reported high percentage of sialic acid in EBN, which is critical in cell communication and signaling. Salicylic acid is also vital for RBC stabilization and in preventing blood component aggregation due to their negative charge and hydrophilicity (Wang et al., 2020). Sialic acids are known to affect the stability and function of hormones and enzymes, they are also known to play a vital role in reproduction, development, and sialylation of follicle-stimulating hormone (FSH) and human chorionic gonadotropin (hCG) and contribute to their stability and function (Yu et al., 2012). Sialic acids are important constituents of glycoprotein and are an important source of nutrients for the growth of ovarian follicles and performs a vital role in ovarian development (Mirni et al., 2014). EBN has been traditionally used as a food that can enhance immunity. EBN supplementation as an antioxidant has been

determined to play a vital role in disease prophylaxis and treatment (Yew et al., 2004; Albishtue et al., 2018).

The dismutation of superoxide results in hydrogen peroxide which lead to formation of hydroxyl radicals that are highly reactive and can modify purines and pyrimidines and cause strand breaks resulting in DNA damage (Agarwal et al., 2005). Previous studies have reported presence of ROS in the female reproductive tract: ovaries (Behrman et al., 2001) and embryos (Guerin et al., 2001). Pb can induce oxidative stress by promoting the generation of reactive oxygen species (ROS) in cells and tissues, which can lead to oxidative damage of cell components such as proteins, lipids, and DNA (Patra et al., 2011). TBARS (thiobarbituric acid reactive substances) indicates an increase in oxidative stress and damage to cellular membranes. TBARS levels were reported to be highest in Pb induced group when compared to R and H EBN treated and negative control group (Esterbauer and Cheeseman, 1990).

MTs are a group of intracellular metal-binding and cysteine-enriched proteins and are highly inducible in many tissues in response to various types of stress (Lu et al., 2004). MT family consists of 4 isoforms: Mammalian MT-1 and MT-2 are present and expressed in almost all tissues (Donald et al., 2015). These proteins are involved in metal detoxification. They can also act as scavengers of ROS and are thought to protect cells against damage from radiation (Adrienne et al., 2006). MT has been reported to protect cells from apoptosis induced by a variety of stimuli including etoposide, oxidative stress, and metals (Baird et al., 2006). MT has been observed in both the cytoplasm and nucleus of LE and GE has been previously reported (Chen et al, 2014), which is another evidence for its role against Pb toxicity. It has been documented that

ROS and oxidative stress play a role in the apoptotic and cytotoxic effects of Pb, which results in the destruction of DNA, proteins and lipid functions (Wang et al., 2009). MTs play an essential role in protecting cells from acute heavy metal poisoning (Park et al., 2001). Gonick et al. (2011) injected lead in metallothionein-null phenotypic mice (mice that have been genetically modified to lack the ability to produce metallothionein) which demonstrate nephromegaly, decreased renal function (Gonick et al., 2011). Albishtue et al. (2020) investigated the protective effects of EBN on kidney and liver tissues against the toxic effects of Pb-toxicity (Albishtue et al., 2020). The study found that EBN protected the kidney and renal tissues from the damaging effects of Pb toxicity due to increased MT-Pb complex formation and excretion, resulting in lower levels of Pb accumulation in the tissues. These finding were further supported by ICP-MS results which showed that Pb deposition in the uterine tissue was highest in positive control group and lowest in the EBN (90 and 120mg/kg) and H EBN (60 mg/kg) groups.

Furthermore, REBN and HEBN may exert their protective effects through the modulation of steroid hormone receptors. Lead acetate exposure can disrupt the normal functioning of steroid hormones, leading to hormonal imbalances and reproductive dysfunction. The observed upregulation of steroid hormone receptors in the presence of REBN and HEBN indicates their potential role in maintaining hormonal balance and preserving normal reproductive function. Moreover, this study revealed that REBN and HEBN possess inherent properties that can mitigate oxidative stress. The evaluation of oxidative stress biomarkers, such as lipid peroxidation and antioxidant enzyme activities, demonstrated the ability of REBN and HEBN to restore the redox balance in reproductive tissues exposed to lead acetate toxicity.

7.5 Conclusion

This study confirms PbA's harmful effects on the reproductive system and indicates that oral supplementation with red (R) and house (H) EBN protects pregnant rats' vital organs. An 8-week R EBN supplementation regimen of 90–120 mg/kg bwt per day and 60 mg/kg H EBN yielded the best results. These doses increased fertility, endometrial receptivity, embryonic implantation, and pinopoded uterine epithelial microvilli. Ebn supplementation improved ovarian interstitial cells, corpus lutea, and atretic follicles. Histologically, uterine glands thickened. Binucleated hepatocytes and normal central veins. Medium-to-high R EBN and H EBN doses restored kidney parenchymal architecture, bowman's capsules, and glomeruli. Immunohistochemistry showed uterine tissues elevated steroid receptors, EGF, EGFR, PCNA and VEGF. R and H EBN medium and maximum dose groups expressed steroid hormones, EGF, EGFR, PCNA densely in ovarian surface epithelium and interstitial cells. ICP-MS showed EBN supplementation dose-dependently reduced PbA accumulation in both tissues. The positive control group exhibited higher MT immunohistochemistry expression in uterine, ovarian, kidney, and liver tissues. Redox imbalance and oxidative stress increased in the positive control group. EBN supplementation protects reproductive and critical organs from PbA. This study shows that R and H EBN increase mammalian reproductive, enabling effective fertility treatments. EBN supplementation's ideal dosage and timing for maximum protection need additional study.

CHAPTER 8

SUMMARY, CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH

8.1 Summary

Indomethacin- and lead-acetate-treated rats were examined for EBN's effects on endometrial receptivity and fertility. Test animals lose fertility to PbA and IMC. This study demonstrated that EBN supplementation may protect the reproductive system from harmful substances. EBN supplementation improved endometrial receptivity, implantation, uterine microvilli, and reproductive indicators. EBN may improve endometrial receptivity, promoting embryo implantation and development. Poor endometrial receptivity causes human and animal infertility.

To demonstrate maternal toxicity, SD rats were administered 4.66 and 5.00 mg/kg bwt IMC on Pd3 and Pd4. An earlier study (Damasceno et al., 2008) found a decrease in feed intake and bwt from Pd6 to Pd11 in rats administered 8.50 mg/kg IMC. The study matches this data. IMC (perhaps a chemical or compound) also negatively affected small intestine smooth muscles, reducing its length (Menozzi et al., 2011). PGs are involved in early implantation like any other inflammatory response (Ricciotti et al., 2011). IMC's toxic injury to uterine tissue may have inhibited endometrial cell PG production, resulting in fewer implantations in treated groups. This may have caused implantation loss in treatment groups (Damasceno et al., 2008). IMC prevents the uterus from producing PG (Sharif et al., 2019).

A degenerative Bowman's capsule and intertubular blood vessel vasoconstriction, dilatation, and congestion were detected histopathologically. Intertubular blood vessels were vasoconstricted. One group of treated SD rats had no pathological alterations other than microvacuolation in the kidney epithelial linings. The control group had higher progesterone levels and more corpora luteum than the other groups, indicating an increase in corpora luteum. Previous research showed that ovarian vasoconstriction indicated blood vessel blockage. No gross clinical or histological abnormalities were identified in vasoconstricted rats' ovaries (Goldenberg et al., 2002). Histological examination demonstrated that microvacuolization of hepatocytes occurred at doses of 5.00 mg/kg and 8.40 mg/kg of IMC, which is entirely consistent with the findings of the previous investigations (Salleh et al., 2014). The findings of the current study demonstrated very clearly that there is an increased density of interstitial cells associated with EBN supplement, which provides further evidence of the significance of EBN supplement in enhancing reproduction and its mechanism of action (Albishtue et al., 2018). VEGF, a protein in EBN, inhibits caspase 3 activation, stimulates rat early antral ovarian follicle cell growth, and prevents cell death. It also helps build ovarian vasculature, allowing nutrients to enter the body. The intervention increased uterine weight and length in both treatment and control groups, according to this study. This result confirmed that EBN oestrogen and sialic acid levels affect uterine growth and length (Albishtue et al., 2019). Histological study of uterine samples from the three treatment groups demonstrated dose-dependent EBN-induced uterine changes. Previous investigations found thicker LE, GE, and endometrium and more uterine glands (Albishtue et al., 2019).

The findings of current study revealed that, in a dose-dependent manner, EBN supplements promote ovarian follicle development, maturity, and survival. EBN may affect the ovary because it contains numerous bioactive compounds. Protein, carbs, ash, and fats are ranked from most to least nutritionally (Ma and Liu 2012a). EBN, renowned for its significant nutritional contribution, contains water-soluble proteins, carbohydrates, iron, inorganic salts, and fiber. Proteins, carbohydrates, and sialic acid comprise EBN. According to Ma and Liu (2012), these three components make up 40–60%, 10–30%, and 6–13% of food item mass (Ma and Liu 2012b).

Histological analysis of ovaries from EBN-treatment groups demonstrated considerable protection, as shown by an increase in gonadotrophic cells, developing follicles, CL, and a decrease in the number of atretic follicles. EBN enhanced ovary weight. PbA-induced uterine weight decrease was seen in this study. Also, histological analysis of the uteri of PbA-exposed rats without the addition of EBN revealed necrosis, a reduction in the number of uterine glands, the destruction of uterine lining cells, and an increase in the filtration of inflammatory cells. This finding is consistent with Eugenia et al. (2009), discovered that despite PbA toxicity, EBN supplemented groups (especially T3) had normal uterine structures and uterine morphological characteristics that were greater than the PbA group and control. EBN's function in cell division, proliferation, and tissue regeneration has also been connected to the existence of an EGF-like component.

This study measured SOD and TBARS levels. The highest treatment group's SOD increased to 90 and 120 mg/kg bwt. The positive control group TBAR concentration was higher than the control and treatment groups.

Lead ions are metalloestrogens because they bind to oestrogen receptors and cause oestrogen-like responses that affect the female reproductive system. Lead exposure during pregnancy may harm enzymes, growth factors, cytokines, and hormones. Preventative medicine, reproductive health, fertility, and health maintenance can use EBN (Filant et al., 2014). PbA has been linked to uterine necrosis (Eugenia et al., 2009). Endometrial glands have been linked to estrous cycles, conceptus survival, and peri-implantation growth (Spencer et al., 2004).

Results showed that EBN had a proliferative effect on GE, LE, and uterine stromal cells as measured by increased expression of EGF, EGFR, VEGF, and PCNA. With regards to PbA toxicity, EBN was both therapeutic and proliferative (Bussmann et al., 2006).

Unlike steroid receptors, oestrogen and progesterone receptors are mostly in the cell nucleus. EBN increased GE and stoma cell P4R expression in this study. Endometrial receptivity requires P4 downregulation during implantation (Abidin et al., 2011; Ma et al., 2012; Albishtue et al., 2019). Lead decays slowly. Lead exposure instantly raises BLL, which then spreads throughout the body. Child bones contain 70% lead. BLL can't compute body burden since lead builds up in bones. This study supports chronic PbA poisoning chelation therapy. Lead is chelated from tissues and blood. Deeply consumed lead may take years to remove from bones (Schroder et al., 2015). These results suggest that the EBN supplement protected against PBA and IMC toxicity in a dose-dependent manner, protecting all pregnancy-related variables (Figure 8.1).

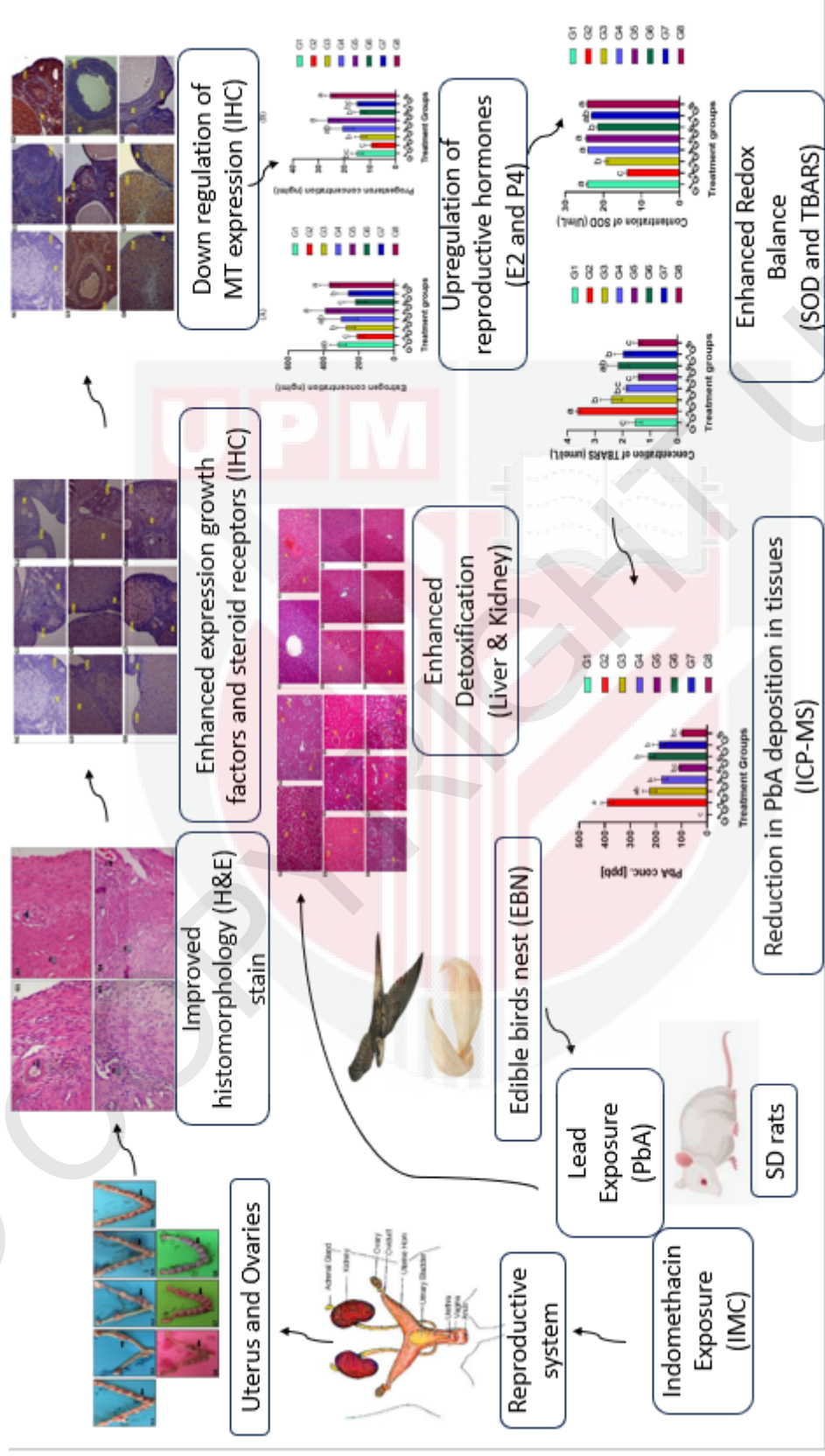


Figure 8.1: Infographic representation of the mechanism of EBN by which it mitigates toxicity effect of IMC and PbA

8.2 Conclusion

In conclusion, these research' findings offer important new understandings of how different treatments and interventions affect rats' reproductive health. The findings show that IMC treatment prevents implantation and embryo development, with Experimental Induced Dysfunction (EID) being induced at a dose of 4.33 mg/kg body weight. In contrast to the kidney tissues' necrosis and the liver tissues' activated Kupffer cells, uterine tissues displayed vasoconstriction and fibrosis.

While IMC therapy caused reproductive toxicity, supplementation with edible bird's nest (EBN) showed promise in protecting against it. Significant improvements in implantation sites and reproductive efficiency were seen at a dosage of 120 mg/kg of EBN. The detrimental effects of IMC on the uterus and ovaries were lessened by EBN supplementation, which also increased antioxidant activity, elevated the expression of growth factors, and decreased the expression of steroid hormones.

Additionally, Red (R) and House (H) EBN oral supplements provided defense against PbA-caused reproductive damage. The development of uterine microvilli and endometrial receptivity were improved as a result of the daily administration of R EBN at dosages of 90 and 120 mg/kg body weight for 8 weeks, along with H EBN at a dose of 60 mg/kg body weight each day. Additionally, the supplementation raised corpus lutea and interstitial cells in the ovaries, decreased atretic follicles, and enhanced the histological characteristics of critical organs.

Overall, these results demonstrate the potential advantages of EBN supplementation in enhancing reproductive health and safeguarding against toxicity brought on by PbA

exposure and IMC therapy. In order to maximize the protective effects of EBN supplementation, more study is required. This will provide important insights for the creation of successful fertility therapies.

8.3 Recommendation for future research

Effects of EBN on pregnant female rats exposed to IMC toxicity were examined in this study; however, to ascertain the effects of EBN on the embryo implantation and foetus development of pregnant rats exposed to IMC, additional research is required.

Effects of R & H EBN on pregnant female rats exposed to PbA toxicity were examined in this study; however, effects of R & H EBN on the embryo implantations of pregnant rats exposed to PbA require further investigation.

Two reproductive hormones, namely E2 and P4, were studied for their response to R & H EBN; further research into the effects of R & H EBN on FSH, LH, and prolactin in rats is needed.

This study only used SOD and TBARS to measure oxidative stress, so more research is needed to determine whether R & H EBN can reduce the oxidative stress caused by PbA on other parameters.

Further long-term PbA exposure research is necessary to understand the protective impact of R & H EBN supplement against PbA toxicity on the female reproductive system of rats.

Molecular studies should be conducted to identify biomarkers responsible for dysfunctional embryo implantation and to determine the extent to which EBN can ameliorate and promote the growth of successful embryo implants.



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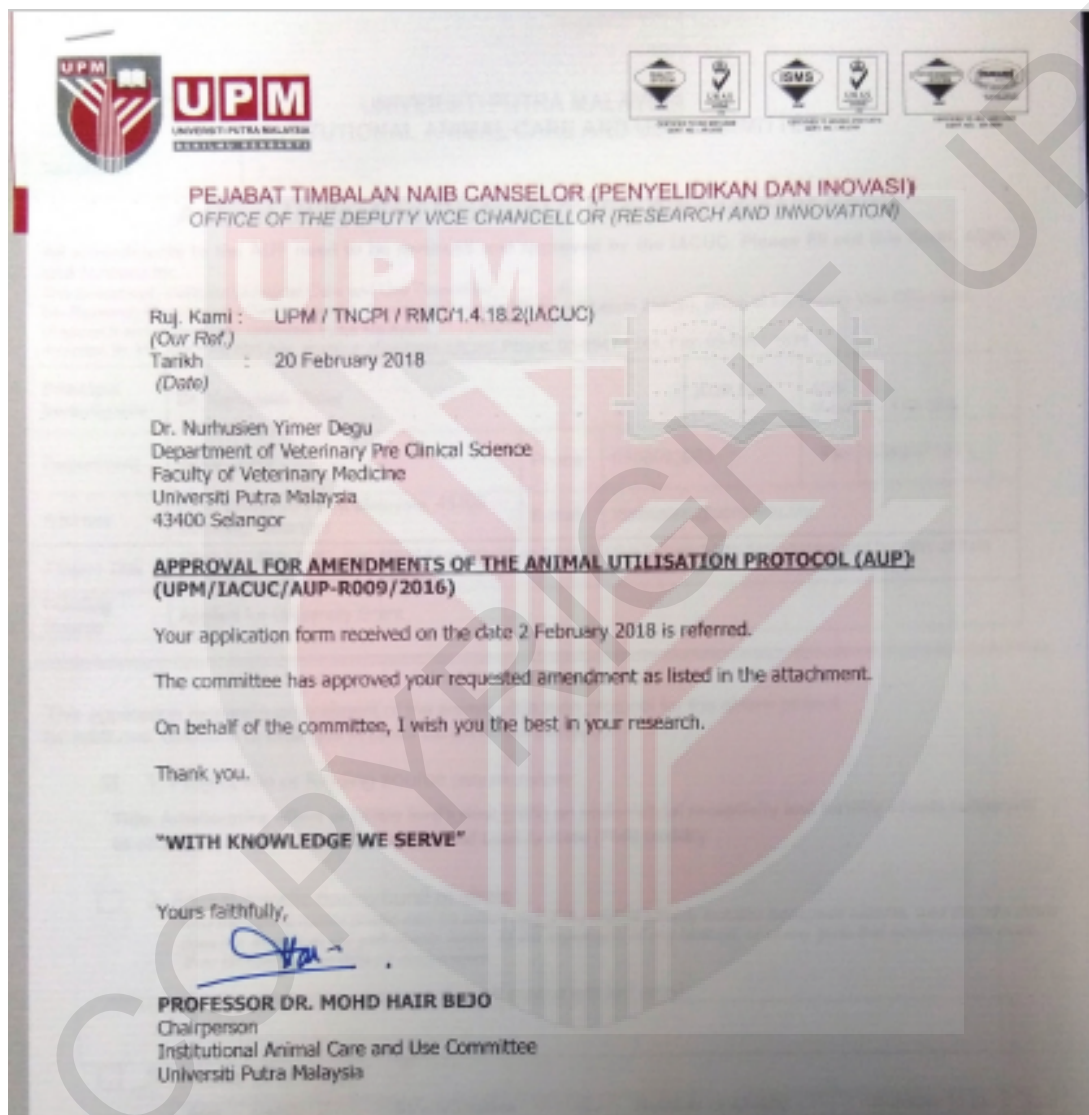
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APPENDICES

Appendix A

Ethical Approval Letter, UPM



Appendix B

Composition of Raw and Hydrolyzed EBN


PERMULAB SDN. BHD.
contributing to nature and environment professionally

WATER | FOOD | MICROBIOLOGY | ENVIRONMENTAL | CONSULTANCY
CO. NO. 392059 K

CERTIFICATE OF ANALYSIS
 REG. NO. : PLF-006132-00010084-18 (1)
 COMPANY NAME : MOBILE HARVESTERS MALAYSIA SDN BHD

PAGE 01 OF 03

 LOI MUN YEE
 SENIOR CHEMIST(M.M.I.C)
 IQM. No. M/3105471.08/11

ATTENTION TO : MS NARGIZA TURATOVA

SAMPLE DESCRIPTION : One (1) no. of Raw Clean EBN

SAMPLE MARKING : Batch No: 1802030650

RECEIVED DATE : 27 April 2018

REPORTED DATE : 08 May 2018

On analysis of the sample(s) submitted, the result(s) obtained as follows:

NO	PARAMETER	METHOD REF. NO.	UNIT	RESULT	**MS SPEC
1	MOISTURE	In-house No F5 (based on AOAC 931.04 by Air Oven Method)	g/100g	14.9	≤15
2	NITRITE calculated as NO ₂	In-house No. P90 (Based on GB5006.33-2016 (IC)	mg/kg	17.4	30 ppm
3	WATER ACTIVITY @25.3°C	In-house No. F53 (Based on Desagor Pw Kt Water Activity Meter)	a _w	0.67	≤1.0
4	AEROBIC PLATE-COUNT	FDA/BAM; Chapter 3	CFU/g	1.3x10 ⁴	≤2.5x10 ⁶
5	YEASTS & MOLDS	FDA/BAM; Chapter 18	CFU/g	ND(<10)	≤ 10
6	COLIFORMS	FDA/BAM; Chapter 4	MPN/g	ND(<1.8)	-
7	<i>Escherichia coli</i>	FDA/BAM; Chapter 4	MPN/g	ND(<1.8)	-
8	<i>Staphylococcus aureus</i>	FDA/BAM; Chapter 12	MPN/g	ND(<3)	≤ 100
9	<i>Salmonella</i>	FDA/BAM; Chapter 5	ix 25g	Absent	Nil
10	PROTEIN/ PROTEIN	In-house No. F7 (Based on AOAC 991.20/ Kjeldahl Method)	g/100g	62.8	-
11	SIALIC ACID	In-house No. F82 (Based on Chinese Journal of Health Laboratory Technology, Mar 2011; Vol 21)	% (w/w)	10.1	Present

Opinion and interpretation expressed herein are outside the scope of SAMM accreditation. This report shall not be reproduced except in full and prior approval of PERMULAB SDN BHD.

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CERTIFICATE OF ANALYSIS

REG. NO. : PLF-006132-00010084-18 (1)

PAGE 02 OF 03

COMPANY NAME : MOBILE HARVESTERS MALAYSIA SDN BHD

NO	PARAMETER	METHOD REF. NO.	UNIT	RESULT	**MS SPEC
12	LEAD as Pb	US EPA 6010B (1996) (ICP-OES) and AOAC 975.03	mg/kg	0.25	2 ppm
13	CADMIUM as Cd	US EPA 6010B (1996) (ICP-OES) and AOAC 975.03	mg/kg	ND (<0.01)	1 ppm
14	ANTIMONY as Sb	US EPA 6010B (1996) (ICP-OES) and AOAC 975.03	mg/kg	ND (<0.01)	1 ppm
15	ARSENIC as As	US EPA 6010B (1996) (ICP-OES) and AOAC 975.03	mg/kg	ND (<0.01)	1 ppm
16	MERCURY as Hg	AOAC 973.21 (Fluorescence AAS Method)	mg/kg	ND (<0.01)	0.05 ppm
17	COPPER as Cu	US EPA 6010B (1996) (ICP-OES) and AOAC 975.03	mg/kg	7.86	1.0 ppm
18	IRON as Fe	US EPA 6010B (1996) (ICP-OES) and AOAC 975.03	mg/kg	3.26	0.3 ppm

NOTE:

1. <- Less Than The Minimum Detection Limit Reported; ND - Not Detected

2. CFU- Colony Forming Unit; MPN- Most Probable Number

3. ** According to MS SPECIFICATION BIRD NEST

METHOD REFERENCE(S):

1. Official Methods of Analysis of AOAC, 16th Edition (1995)

2. The Chemical Analysis Of Foods, 7th Edition, David Pearson (1976)

3. U.S Environmental Protection Agency Method (EPA) Revision 2, 1996.

4. Food and Drug Administration/ Bacteriological Analytical Manual (FDA/BAM) Online; January 2001., August 2001, September 2002, January 2003 & June 2006.

5. Ion Chromatography (IC)

6. National Food Safety Standard: Determination of Nitrate and Nitrite in Foods; Implemented on 2010-06-01; Published by Ministry of Health of the People's Republic of China.

7. Chinese Journal of Health Laboratory Technology, Mar 2011; Vol 21


 LOI MUN YEE
 SENIOR CHEMIST(M.M.I.C)
 ICM. No. M/3310/5471/08/11

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Composition of Hydrolyzed EBN



Ref : ULUKM/1338/17

Date : 29/03/2017

Page : 1 of 1

CERTIFICATE OF ANALYSIS

Name of Customer : **SBNB SDN BHD**
 Address : **Makmal MANIS**
 Universiti Kebangsaan Malaysia,
 43600 Bangi, Selangor

Tel. No. : 014-5115159

Sample Description : One sample described as **BIOACTIVE EBN HYDROPEPTIDE**

Sample Ref. No : U0887/17

Date of Receipt : 09/03/2017

ANALYSIS RESULTS (As per sample)

Parameter, Unit	Result	Test Method
Protein, g/100g	64.0	In house method No. STP/Chem/A03 based on AOAC 16 th Edi. 961.10
Total Fat, g/100g	0.6	In-house Method No: STP/Chem/A02(i) based on The Chemical Analysis of Food, David Pearson, 6th Edition: Page 13
Total Carbohydrate, g/100g	26.6	In house method No. STP/Chem/A05 based on Promerance Food Analysis: Theory and Practice, 2nd Ed. (pg 637)
Total Ash, g/100g	2.2	In house method No. STP/Chem/A05 based on AOAC 16 th Edi. 923.03
Moisture, g/100g	6.6	In house method No. STP/Chem/A04 based on AOAC 16 th Edi. 950.46
Energy, kcal/100g	368 (1546kJ)	In house method No. STP/Chem/A01 based on Pearson's The Chemical Analysis of Foods (6th Edition, page 578)

Remarks:

- %Total Carbohydrate = 100 - (%Ash + %Moisture + %Protein + %Fat)
- Opened / balance samples will be discarded two weeks after issuance of Certificate of Analysis.


 NOOR AZIDAH MAAMIN
 CHEMIST (M.M.L.C.)
 IKM No. M/1973/4360/03



This report refers to the tested sample only. Sampling is not carried out by our organization. All analysis are carried out to the best of our knowledge and ability and our responsibility is limited to the correctness of the result. This report is issued on the understanding that it does relieve parties concerned from their contractual obligations. This report shall not be reproduced except in full without written approval of the laboratory.

**Bridge To A
Better Product**

UNIPEQ Sdn. Bhd. (870956-D) Block A, UKM - MTDC Technology Centre, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia.

Tel : 603 8921 5985

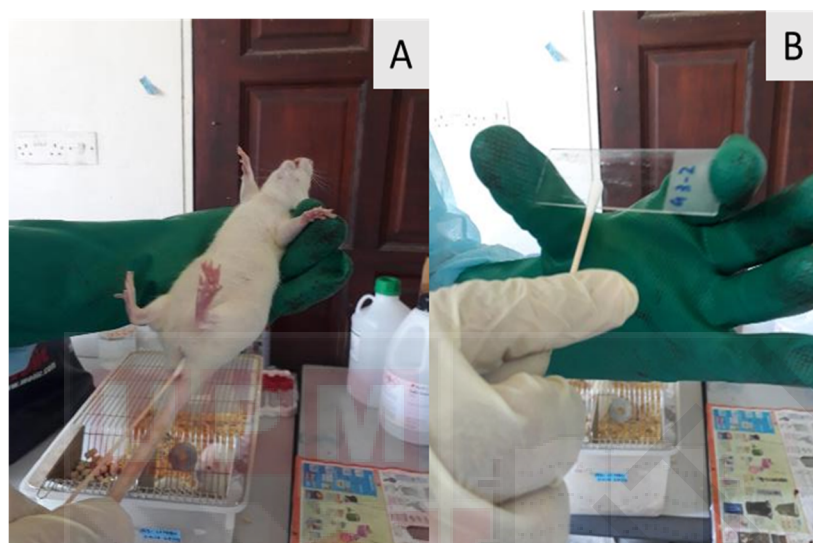
Fax : 603 8925 2115

Email : unipeq@ukm.edu.my

Web : www.unipeq.com.my

Appendix C

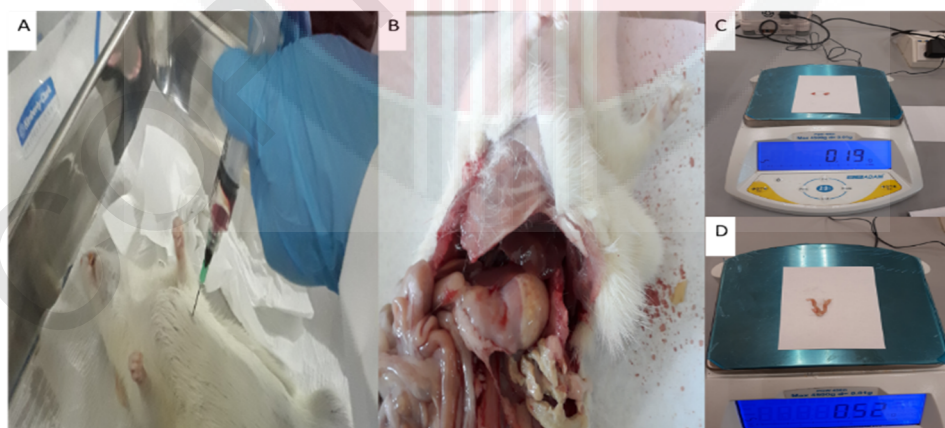
Vaginal Smears to observe oestrous cyclicity



Vaginal swab sampling (A) and preparation of smear on a microscope slide (B)

Appendix D

Rat under general anaesthesia sacrificed, blood collection, ovary, and uterine tissue harvesting



A: Blood collection from anesthetized rat via cardiac puncture B: Abdomen dissection of rat C: Weighing Ovaries D: Weighing Uterus

Appendix E

ELISA procedure for E2

Reagent preparation

One standard tube (labelled as tube zero) was supplemented with 1 ml of sample dilution buffer to prepare the standard solution. The tube was then left at room temperature for 10 minutes while being thoroughly mixed. Labelled seven Eppendorf (EP) tubes with the following designations: 12, 14, 1/8, 1/16, 1/32, 1/64, and blank. Each tube was filled with 0.3 ml of the sample dilution buffer. 0.3 cc of the aforementioned standard solution were added to the first tube and carefully mixed. I added 0.3ml from the first tube to the second tube and properly stirred. transferred and mixed 0.3 ml from the second tube into the third, and so forth. The blank control used a sample dilution. Calculate the total volume of the working solution needed for the synthesis of the biotin-labeled antibody using the formula: $0.1 \text{ ml/well} \times \text{the number of wells}$ (allowed 0.1-0.2 ml more than total volume). The biotin-detection antibody was well mixed and diluted with an antibody dilution buffer at a ratio of 1:100. Working solution for the conjugation of HRP and streptavidin was created by estimating the volume of the solution. The HRP- streptavidin was fully mixed after being diluted 1:100 with the dilution buffer.

Assay procedure

Before adding the standard, sample, and control (blank) wells, wash the plate twice. On the pre-coated plate, place standard, test samples, and control (blank) wells, in that order, and then note their locations. 50ul of the standard, a blank, or a sample were added to each well. Standard buffer solution was used to add the blank. Each well was

immediately filled with 50ul of biotin-labeled antibody working solution. sealed with a plate covering. 45 min. of incubation at 37°C. Following incubation, rinse the plates three times with wash buffer, allowing each rinse to sit in the wells for one minute. Remove any leftover wash buffer after the last wash by aspirating or decanting. Then, 100ul of the HRP working solution was added to each well. I used a fresh Plate sealer to cover it. 30 min. of incubation at 37°C. Wash the plate with wash buffer five times after incubation. The addition of 90ul of TMB substrate solution. 10–20 minutes at 37°C of incubation. 50ul of stop solution was then added. The colour immediately turned yellow. After applying the stop solution, immediately read the optical density (OD) absorbance at 450 nm in a microplate reader.

ELISA procedure for P4

Reagent Preparation

Before use, bring all reagents to room temperature (18–25 °C). 30 mL of concentrated wash buffer was diluted with 720 mL of distilled water to create 750 mL of wash buffer. Centrifuge the standard at 10,000 rpm for 1 minute to prepare the working standard. A pipette was used to completely combine the sample diluent with the reference standard and add 0.1 mL of each. Then, where required, serial solutions were produced. Method of dilution: 7 EP tubes were used, and the first tube received 500ul of the working solution before being fully mixed. Following these steps, pipet 500uL from the former tube into the latter. Prior to the experiment, determine the necessary quantity for the working antibody solution (50ul/well). Biotinylated Detection Ab diluent was used to dilute the 100X concentrated Biotinylated Detection Ab to 1X working solution. Additionally, the required amount of HRP conjugate (100 uL in each well) was produced.

Assay procedure

The first two columns were filled with the standard working solution, which was added in duplicate, side by side, to each well (50 uL for each well), at each concentration. 50 uL of working antibody solution was added to each well immediately after adding the samples to the other wells. applied sealant to the plate. The plate was incubated for 45 minutes at 37C. After incubation, remove the solution from the wells and fill each one with 350 uL of wash buffer. Let the wells soak for 1-2 minutes before aspirating the wash buffer. Each well received 100 uL of the HRP conjugate working solution, which was then incubated at 37°C for 30 minutes. Following incubation, remove the solution from each well and carry out the wash procedure five more times. After that, each well received 90uL of substrate reagent before being sealed with fresh plate sealer. shielded the plate from light and incubated the plate for approximately 15 minutes at 37C. 50uL of stop solution were thereafter added to each well. Utilised a microplate reader set to 450 nm to calculate the OD of each well.



Elisa procedure for analyzing E2 and P4 serum concentration

Appendix F

H&E staining procedure for microscopical examination

Method

- Tissue sections were collected (4 µm) which were cut from paraffin-embedded blocks on clean glass slides.
- Dewaxed through graded alcohols followed by rinsing the slides into running water.
- Stained in Haematoxylin for 5 minutes.
- Dipped the slides 2-3 times in 1% acid alcohol. Washed in running water until turned blue.
- Stained in eosin for 30 sec to 1 minute.
- Dehydrated through alcohol series and cleared in xylene.
- Mounted with a coverslip using DPX and left to dry.

Giemsa staining for Vaginal cytology.

- Dipped the smear (3-4 dips) into pure methanol for fixation of the smear, left to air dry for 1 minute.
- Stained the slide with 5% Giemsa stain solution for 10-20 minutes.
- Washed with tap water and left to air dry.

Appendix G

Immunohistochemistry protocol for rat uterine and ovarian tissues

Mounting and Deparaffinization of Tissue Sections:

- Collected sectioned (4 µm) tissues cut from paraffin-embedded blocks on clean glass slides.
- Rehydrated through graded alcohols followed by rinsing the slides into running water.
- Stained in Haematoxylin for 5 minutes.
- Dipped the slides 2-3 times in 1% acid alcohol. Washed in running water until turned blue.
- Stained in eosin for 30 sec to 1 minute.
- Dehydrated through alcohol series and cleared in xylene.

Target Retrieval (Heat-Induced Epitope Retrieval)

Prior to use, the product was diluted 1:10 with distilled water.

(Nine parts water) to make a working solution.

1. Deparaffinized and rehydrated sections of tissue.
2. Immersed sections in suitable containers filled with diluted Target Retrieval Solution.
3. Performed antigen retrieval (10 minutes in high power for microwave with minimum volume 500-700ml of antigen retrieval.
4. Decanted Target Retrieval Solution and rinsed sections 2 to 3 times with room temperature buffer

Procedural Notes

STEP 1 PEROXIDASE BLOCK

1. Tapped off excess wash buffer. Using a lintless tissue, carefully wiped around the specimen to remove any remaining liquid and to keep reagent within the prescribed area.
2. Applied enough Peroxidase Block to cover specimen.
3. Incubated for 10 minutes (± 1) minutes.
4. Rinsed gently with distilled water or wash buffer from a wash bottle (do not focus flow directly on tissue) and place in a fresh buffer bath.

STEP 2 PROTEIN BLOCK

1. Tapped off excess buffer and wiped slides as before.
2. Applied enough protein block to cover specimen.
3. Incubated to 5 (± 1) minutes.
4. Rinsed gently with buffer solution from a wash bottle.

STEP 3 PRIMARY ANTIBODY OR NEGATIVE CONTROL REAGENT

1. Tapped off excess buffer and wiped slides as before.
2. Applied enough optimally diluted primary antibody or negative control reagent to the covered specimen.
3. Incubated at room temperature for overnight at 4 degrees.
4. The next day, rinsed gently with a buffer solution from a wash bottle and placed it in a fresh buffer bath.

STEP 4 PEROXIDASE LABELLED POLYMER

1. Tapped off excess buffer and wiped slides as before.
2. Applied enough Apply HRP-conjugate to cover specimen.
3. Incubated 15 ($\pm$ 1) minutes. Rinsed slides as in Step 2

STEP 5 DAB+ SUBSTRATE-CHROMOGEN

1. Wiped slides as before. Added 30 μ l (1 drop) DAB Chromogen to 1.5 ml (50 drops) of DAB Substrate, mixed by swirling and applied to the tissue.
2. Applied enough prepared DAB+ substrate-chromogen to cover specimen.
3. Incubated for 10 ($\pm$ 1) minutes.
4. Rinsed gently with distilled water from a wash bottle.

STEP 6 HEMATOXYLIN COUNTERSTAIN (optional)

1. Immersed slides in a bath of hematoxylin. The length of incubation depends on the strength of hematoxylin used. Rinsed gently in a distilled water bath.
2. Rinse slides in a bath of distilled or deionized water for 2–5 minutes.

STEP 7 MOUNTING

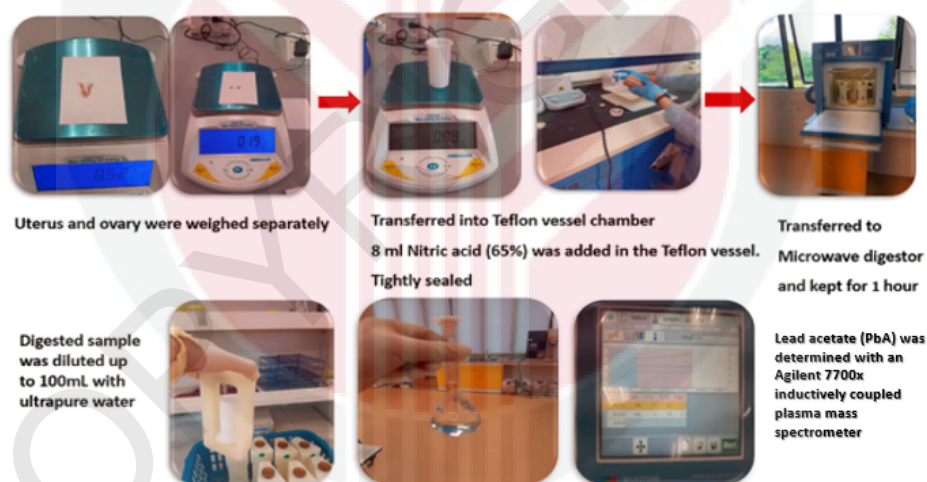
1. Specimens were mounted and cover slipped with an aqueous-based mounting medium.

Appendix H

Sample preparation of ICP-MS

Steps

1. Uterus and ovary were weighed separately.
2. Transferred into Teflon vessel chamber.
3. ml Nitric acid (65%) was added in the Teflon vessel.
4. Vessels were tightly sealed.
5. Vessels transferred to Microwave digester and kept for 1 hour.
6. After 1 hour cleaned digested solution was obtained, it was diluted up to 100mL with ultrapure water.



Steps involved in sample digestion (ICP-MS)

Appendix I

Histopathological lesion scoring

	Fields						
Organs/Scoring	1	2	3	4	5	6	Mean
Uterus							
Ovaries							

Histological scoring

Scoring Method	0 (Normal)	1(Mild)	2 (Moderate)	3 (Severe)
Histopathological changes	00	<25	<50	>50

BIODATA OF STUDENT

The student was born on January 25, 1990, in Hyderabad District, Sindh Province, Pakistan. She attended primary school at Yesgol Heiral, Bangor, UK from 1994 to 1997 and secondary school at St: Bonaventure's High School from 1998 to 2005. From 2005 to 2007, she completed secondary school at Preston Girls College. In 2009, she was admitted to study Veterinary Medicine at Sindh Agriculture University. In 2014 she graduated with top grades in clinical medicine, theriogenology, veterinary pathology, and animal nutrition. She received a fully funded master's scholarship, persuaded her to go to US, and received her Master in animal biotechnology and biomedical sciences, which she completed in 2017. She has been involved in research activities since her senior year at the university. Therefore, upon graduation, he was selected as a research officer in the Ministry of National Food Security and gained diverse experience in research-related activities in the fields of agriculture and animal sciences. The student pursued her doctorate in philosophy from UPM, Malaysia and was admitted in theriogenology and cytogenetics. Research focused on "Effect of edible bird's nest on endometrial receptivity and fertility of rats subjected to indomethacin and lead acetate toxicity"

LIST OF PUBLICATIONS

Maria Amir, Nurhusien Yimer., Mark Hiew, Sabri Mohd Yusoff, Bedru Hussen and Quddus Abdul., 2023. Effect of indomethacin on embryo implantation and histomorphology of uterus, ovary, kidney, and liver of rats. *Saudi Journal of Biological Sciences*, 30(12), 103837. (Accepted)

Maria Amir, Nurhusien Yimer., Mark Hiew, Sabri Mohd Yusoff, Sadiq Mohammed Babatunde and Quddus Abdul., 2024. Edible Birds Nest (EBN) Ameliorates the Effects of Indomethacin (IMC) induced embryo implantation dysfunction in rats. *Animal MDPI*. (Under review.)

Conferences

Maria Amir, Nurhusien Yimer, Mark Hiew, Sabri MY, Quddus Abdul. (2019) Prophylactic effect of EBN on uterine function of cycling female rats subjected to cadmium toxicity. 31st Veterinary Association Malaysia Congress 2019, Bangi Resort, Malaysia. Oral presentation.

Maria Amir, Nurhusien Yimer, Mark Hiew, Sabri MY, Quddus Abdul. (2022) Edible Birds Nest (EBN) therapy against indomethacin induced reproductive dysfunction in rats. Proceedings of the 41st MSAP Annual Conference & 6th ARCAP Bayview Hotel, Georgetown, 26th – 28th October 2022.

Maria Amir, Nurhusien Yimer, Mark Hiew, Sabri MY, Quddus Abdul. (2023) Prophylactic effect of EBN on uterine function of cycling female rats subjected to cadmium toxicity. 31st Veterinary Association Malaysia Congress 2019, Bangi Resort, Malaysia. Oral presentation.