

**EFFECTS OF CHRONIC ADMINISTRATION OF
NIFEDIPINE ON THE REPRODUCTIVE SYSTEM OF
WISTAR RATS**

BY

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MAY, 2018

CERTIFICATION

We, the undersigned, hereby certify that **ABIOYE**, Adesola Idowu Abdur-Raheem carried out this research work under our supervision in the Department of Anatomy, Faculty of Basic Medical Sciences, College of Health Sciences, University of Ilorin, Ilorin, Nigeria.

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DEDICATION

This work is dedicated to Almighty Allah (S.W.T.) for His continuing guidance and protection over my life.

ACKNOWLEDGEMENT

My ultimate gratitude goes to Almighty Allah (S.W.T), who has seen me through the conception, execution and completion of this programme. AL-HAMDULILLAHAH!.

I thank the University of Ilorin Administration for the Staff Development Award I obtained to undertake my Ph.D. programme and the understanding and support throughout the period of the programme.

My profound and immeasurable gratitude goes to my ever - supportive and dynamic supervisors, Dr. M. S. Ajao for giving their time to read through this work, making constructive criticisms and giving useful directions to ensure that this work is successful not forgetting their encouragement, word of advice and support at each stage of this work. You are both appreciated! I use this medium to appreciate the contribution of Late Professor E. A. Caxton-Martins, who started the supervision of this work, prior to his demise. Similar gesture is extended to my then co – supervisor, Prof. C. N. B. Tagoe, while he was here as a full-time staff.

This study could not have been achieved without the moral and intellectual input of the Head of the Department and the various postgraduate coordinators, Drs. M. S. Ajao, O. B. Akinola, A. O. Oyewopo, G. O. Omotoso and A. S. Alabi respectively. I also appreciate the intellectual input of other colleagues in the Department, viz: Dr. B. U. Enaibe, Dr. A. A. Akinlolu, Dr. R. E. Kadir, and the former H. O. D. , Prof. S. B. Agaja. I commend the invaluable of the senior members within the Faculty of Basic Medical Sciences, especially Prof. A. O. Soladoye, Dr. B. V. Owoyele, Dr. L.A. Olayaki, Dr. L. A. Olatunji, Dr. S. A. Biliaminu and other academic staff members. A special mention should be reserved for the invaluable moral and intellectual contributions and time given by Dr. A. B. Nafiu of the Department of Physiology.

The technical staff of the Department of Anatomy as well as the sister departments were also very supportive. I wish to use this opportunity to appreciate their sacrifice, commitment and assistance towards the completion of the work. Such persons include: Mr. E. O. A. Abiola, Mr. P. O. Awolola, Mr. J. T. Adetoro, Mrs. M. F. Adigun, Miss. S. F. Lewu, Mr. S. Danlami and Mr. M. Adewole in the Laboratories. The moral and technical support of sister laboratories' staff members cannot be overlooked; such include: Mr. A. Tijani, Dr. M. K. Bello, Dr. G. O. Adunmo, Mrs. F. O. Oni, Mr. A. T. Sanni, Mrs. Sanni, Mrs. Olawale-Bello and Mr. O. Adesua.

I must appreciate Prof. S. O. Salami, of the Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Ilorin, Ilorin, for linking me up with the technical staff of the Departments of Veterinary Physiology and Veterinary Histopathology unit. The technical advice given by Mr. R. Suleiman and the use of Automatic Tissue Processor by Mr. A. A. Adegboye would ever be remembered!

I must also not forget the immense support of Messrs A. F. Olalere and L. O. Olatunbosun, both of the Department of Haematology, University of Ilorin Teaching Hospital, Ilorin, Nigeria, and Mr. A. Fowotade of the Histopathology unit of the same institution. The latter helped me in carrying out immunocytochemical studies on my specimens.

At this juncture, I wish to appreciate the efforts by Messrs M. J. Suleiman, A. O. Aleshinloye, T. O. Ajao, K. A. Akinyemi and U. O. Yahaya for all the moral support and assistance rendered, as well as other Anatomy, Physiology and medical students who contributed in one way or the other to this study.

I am also grateful to members of my immediate and extended families; wives and children, for their understanding, tolerance and care, moral, tactical and spiritual support throughout the course of the programme. They are the covering garments for my nakedness!

My appreciation also goes to my beloved kiths and kins, friends and dependable associates; Dr. M. S. Tokede, Barr. N. Salman-Mann, Chief (Arc.) A. O. Olaniyi, Chief (Barr.) M. B. Olaniyi, Al-Hajj A. A. Abioye, Al-Hajj L. S. Abioye, Profs. B. L. Adeleke, A. A. Adesokan and Al-Hajj I. A. Alao, whose love and care have been keeping me going.

May the Almighty Allaah richly reward all with His limitless bounties, aamiina!

DECLARATION

I, ABIOYE, Adesola Idowu Abdur-Raheem, hereby declare and confirm that the work presented here is my own. Where information has been derived from other sources, I confirm that it has been indicated clearly.

A. I. 'R. Abioye

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ABSTRACT

Nifedipine, one of the antihypertensive drugs is associated with various side effects on organs of the body but there is paucity of information on its effects on female reproductive system of Wistar rats. The study evaluated the effects of Nifedipine on the female reproductive system. The objectives of the study were to: (i) investigate the effect of Nifedipine on the oestrous cycle of the non – pregnant Wistar rats; (ii) determine the effect of Nifedipine on the serum levels of oestradiol, progesterone, and gonadotrophins in non - pregnant and pregnant Wistar rats; (iii) investigate the effect of Nifedipine on the foetuses at term; and (iv) demonstrate any histoarchitectural changes in the ovary of the treated animals.

The study was carried out in two experimental stages: in the first stage, eighteen cycling non-pregnant Wistar rats were used. They were divided into three groups with each group comprising six rats. Rats in group I were administered 10 ml/kg body weight of distilled water (control), group II was administered aqueous solution of Nifedipine 1.5mg/kg body weight, while rats in group III were administered 3 mg/kg body weight. In the second stage, eighteen pregnant Wistar rats were used. They were grouped and treated as the cycling non-pregnant Wistar rats. Doses of distilled water and the drug solutions were delivered orogastrically once daily for 21 days. Vaginal smears cytology was done daily throughout the 21 days in the non-pregnant rats and the cell types recorded. All the medications were administered through the orogastric route. At the end of the experiment, the rats were euthanized using 20 mg/kg body weight of ketamine hydrochloride. Whole body perfusion fixation was done using 4% paraformaldehyde. Blood specimens were taken for evaluation of sex hormones. The ovaries were removed and fixed in 4% paraformaldehyde solution before tissue processing. In the pregnant groups, the pup-containing uteri were similarly harvested

for morphological evaluation. Data were analysed using Student's t-test at 0.05 level of significance.

The findings of the study were that:

- (i) there was total disruption of the oestrous cycles in Nifedipine treated in both the low and high-doses compared with the controls;
- (ii) the serum levels of oestradiol was 41.0 ± 6.14 pgm/ml for the control, 75.83 ± 12.61 pgm/ml for non-pregnant and 2036.0 ± 50.57 pgm/ml for the pregnant rats;
- (iii) luteinizing hormone concentration was 13.6 ± 5.51 mIU for non-pregnant, 5.9 ± 0.64 mIU for the pregnant and 9.48 ± 1.45 mIU for the control;
- (iv) the serum levels of progesterone was 26.2 ± 2.33 ng/ml in non-pregnant rats, 98.4 ± 1.47 ng/ml in pregnant and 24.2 ± 0.96 ng/ml in the control;
- (v) there were no obvious congenital malformation affecting the foetuses; and
- (vi) there was loss of cortico-medullary demarcation in the treated groups.

The study concluded that there were structural ovarian changes associated with use of Nifedipine in Wistar rats. The study recommended that Nifedipine should be avoided in control especially in non-pregnant individuals.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

High blood pressure (hypertension) refers to a clinical state where an individual is said to be having a prolonged and sustained elevation of blood pressure (BP) above the normal range for age, sex and race. The normal range is from 100 to 140 millimetre of mercury (mmHg) for systolic BP and 60 to 90 mmHg for diastolic BP. Hence, the diagnosis of hypertension is made if and when the systolic BP persistently exceeds 140 mmHg or the diastolic BP persistently exceeds 90 mmHg (Abalos *et al.*, 2007). The risk of developing high blood pressure increases as the level of blood pressure increases. The development of complications of hypertension often arises when left untreated or not properly controlled resulting in stroke, coronary heart disease and renal failure. Patients with high blood pressure must take their antihypertensive drugs regularly and for a very long time and in most cases for life (Redwood, 2007). Although the drugs cannot give a permanent cure, they have the ability to the development of complications (Abalos *et al.*, 2007).

It has been documented in the literature that medications have effects on various organs of the body without any review on the female reproductive system particularly on the ovary. Many people, especially women, engage in the use of antihypertensive drugs without knowing the effects of those drugs on their reproductive system. Hypertension is a chronic medical condition in which the blood pressure in the arteries is elevated (Redwood, 2007). This requires the heart to work harder than normal to circulate blood through the blood vessels. Antihypertensives are classes of drugs that are used to treat hypertension (Redwood, 2007).

Antihypertensives drugs are classified into different classes based on the mechanisms of action and their ability to lower the blood pressure. Relevant examples are angiotensin-

converting enzyme (ACE) inhibitors, thiazides, centrally-acting sympathetic inhibitors, the calcium channel blockers (or inhibitors), peripherally-acting sympathetic inhibitors, vasodilators, angiotensin II receptors antagonists. Antihypertensive drugs that are commonly used can be classified into three categories: (i) Diuretics: they lower blood pressure by increasing urination. Common examples are hydrochlorthiazide and indapamide. (ii) Vasodilators: they dilate blood vessels to lower blood pressure. One common example is nifedipine. (iii) Other drugs: these achieve an effective control of the level of blood pressure by regulating bodily functions that governs blood pressure. Common examples are metoprolol, propranolol, methyldopa and captopril.

Calcium channel blockers (CCB) or inhibitors are also termed “calcium channel antagonists”. This refers to a chemically, pharmacologically and therapeutically heterogeneous group of drugs prominent both as cardiovascular therapeutic (Epstein *et al.*, 2002). The cardiovascular activities of these drugs as antihypertensive, antianginal and selective antiarrhythmic agents are due to their interaction at one particular calcium mobilization process—calcium entry through a voltage-gated calcium channel of the L-type.

Many studies have demonstrated that, in accordance with their chemical heterogeneity, these agents interact at discrete receptor sites associated with a major subunit of the channel. The properties of these drugs and the mechanisms by which they function have been extensively reviewed (McDonough, 2004).

The CCB class is easily identified by the suffix "-dipine". Amlodipine, Aranidipine, Azelnidipine, Barnidipine, Benidipine, Cilnidipine, Clevidipine, Isradipine, Manidipine, Nicardipine Nifedipine, Nilvadipine, Nimodipine, Nisoldipine, Nitrendipine, Pranidipine. All of these drugs act by blocking voltage-gated calcium channels in (largely) arterioles and in

cardiac muscle, resulting in vessel relaxation (negative inotropic effect) and decreased heart rate (negative chronotropic) and contractility.

Nifedipine is a dihydropyridine calcium channel blocker. Its main uses are as an antianginal (especially in Prinzmetal's angina) and antihypertensive, although a large number of other indications have recently been found for this agent, such as Raynaud's phenomenon, premature labour, and painful spasms of the oesophagus such as in cancer and tetanus patients. It is also commonly used for the small subset of pulmonary hypertension patients whose symptoms respond to calcium channel blockers (Levin *et al.*, 1994).

Ovarian disorders that often arise from effect(s) following use of different drugs may affect the activities of the gonads by disrupting the hypothalamic – pituitary – gonadal axis. Studies have shown that antineoplastic drugs and hormonal therapy are of major concerns. Some antibiotics and neuroleptic agents can also cause endocrine disorders (Buchanan & Davis, 1984). It has been documented in the literature that medications have effects on various organs of the body without any review on the female reproductive system particularly on the ovary. Many people, especially women, engage in the use of antihypertensive drugs without knowing the effects of those drugs on their reproductive system.

Though, hypertension is a chronic medical condition in which the blood pressure in the arteries is elevated (Redwood, 2007), it is an important worldwide public-health challenge because of its high frequency and concomitant risks of cardiovascular and kidney disease. It has been identified as the leading risk factor for mortality and ranked third as a cause of disability-adjusted life-years (Manimunda *et al.*, 2011; Udenigwe *et al.*, 2011).

1.2 Statement of the problem

There is paucity of studies of the effects of commonly - used antihypertensive drugs on the female reproductive system such as how they affect the menstrual cycle, ovulation,

fertility and changes in the serum concentrations of ovarian hormones. Most recent studies attempt to look at the activities of chronic administration of some antihypertensives (clonidine, methyldopa, amlodipine, ramipril and rilmenidine) on the enzymatic, non-enzymatic effects on the oxidative and non-oxidative parameters on the ovarian tissue (Salman *et al.*, 2011). The activities and effects of these drugs on the hypothalamic-pituitary-gonadal pathways were not studied and this is key to the understanding of their effects on such pathways since it can explain some unexplained symptoms in female patient on chronic antihypertensives. There is the need to define these effects, if any, in terms of the changes in the ovarian functions such as hormonal, the oestral cycle, ovulation and oogenesis. These will add pragmatic and measurable values to the choice of the drugs in the treatment of hypertension particularly in the females. There is also the need to identify if the effects are reversible with subsequent withdrawal of the drugs.

1.3 Justification for the study

Despite the need for the universal use of nifedipine, there is a dearth of information on the consequences of its immediate and prolonged use on the ovary. This study is undertaken to bring about the effects of nifedipine on the ovary structurally and functionally. The results from this research would strengthen the safe use of nifedipine without fear of any adverse effects on the ovary.

1.4 Significance of the study

Nifedipine are commonly used for management/treatment of various types of hypertension; their effects have been studied and reported on various organs of the body but there is a dearth of such researches on the female reproductive system. This study was carried out to

bridge the knowledge gap regarding existence of any possible deleterious or salutary effects resulting from the use of nifedipine on the ovary that could be avoided or encouraged.

1.5 Aim of the Study

The aim of this study is to determine the effects of chronic administration of *nifedipine* on the histoarchitecture and functions of the ovary of adult Wistar rats.

1.6 Specific Objectives

The specific objectives of the study are to:

1. investigate the activities of nifedipine on the oestrous cycle of the non – pregnant and pregnant Wistar rats;
2. determine the efficacy of nifedipine on the serum levels of oestradiol, progesterone, gonadotrophins in both non - pregnant and pregnant Wistar rats;
3. investigate the effects of nifedipine on the foetuses at term; and
4. demonstrate any histoarchitectural changes in the ovary of the treated rats.

1.7 Scope of the study

This research work is limited to:

- (i) assessment of the morphological changes in the treated animals;
 - (ii) evaluation of the changes in the oestrous cycles;
 - (iii) assay of the serum levels of the gonadotrophic hormones, oestrogen and progesterone;
- and,

(iv) the morphological changes observable in the ovaries and the products of gestation, where applicable, using anthropometric, histological and immunohistochemical techniques.

(v) Evaluation of the haematological parameters and the effect of *Nifedipine* on the histoarchitecture of the ovary in non – pregnant and pregnant Wistar rats.

1.8 Research Hypothesis

Chronic administration of nifedipine alters oestrous cycle, ovarian morphology, foetal morphology and alters serum levels of gonadotrophins, oestrogen and progesterone in female Wistar rats.

1.9 Limitations

A few limitations were encountered. These included inability to get appropriate laboratory spaces; paucity of appropriate chemicals, reagents, paucity of modern laboratory equipment and poor access to funding.

CHAPTER TWO

2.0 LITERATURE REVIEW

The risk of developing high blood pressure increases as the level of blood pressure increases. The development of complications of hypertension often arise when left untreated or not properly controlled resulting in stroke, coronary heart disease and in some cases, renal failure. High blood pressure (Hypertension) refers to a clinical state where an individual is said to be having a prolonged and sustained elevation of blood pressure above the normal range for age, sex and race. The normal range is from 100 to 140 mmHg for systolic BP and 60 to 90 mmHg for diastolic BP. Hence, the diagnosis of hypertension is made if and when the systolic BP persistently exceeds 140 mmHg or the diastolic BP persistently exceeds 90 mmHg (Abalos *et al.*, 2007). Patients with high blood pressure must take their antihypertensive drugs regularly and for a very long time and in most cases for life (Redwood, 2007). Although the drugs cannot give a permanent cure, they have the ability to delay the development of complications (Abalos *et al.*, 2007).

It has been documented in the literature that medications have effects on various organs of the body without any review on the female reproductive system particularly on the ovary. Many people, especially women, engage in the use of antihypertensive drugs without knowing the effects of those drugs on their reproductive system. Hypertension is a chronic medical condition in which the blood pressure in the arteries is elevated (Redwood, 2007). This requires the heart to work harder than normal to circulate blood through the blood vessels. Antihypertensives are classes of drugs that are used to treat hypertension (Redwood, 2007).

2.1 The Calcium Channel Blockers [CCB]

These are “calcium channel antagonists”; they are also called calcium ion influx inhibitor, slow-channel blocker or calcium ion antagonist. They are a chemically, pharmacologically and therapeutically heterogeneous group of drugs (Epstein, 2002).

Their cardiovascular activities (i.e. as antihypertensive, antianginal and selective antiarrhythmic agents) are due to their interaction at one particular calcium mobilization process—calcium entry through a voltage-gated calcium channel of the L-type. These agents interact at discrete receptor sites associated with a major subunit of the channel (McDonough, 2004).

They are easily identified by the suffix "-dipine" - e.g. Amlodipine, Aranidipine, Azelnidipine, Barnidipine, Benidipine, Cilnidipine, Clevidipine, Isradipine, Manidipine, Nicardipine, Nifedipine, Nilvadipine, Nimodipine, Nisoldipine, Nitrendipine, Pranidipine. All of them act by blocking voltage-gated calcium channels in (largely) arterioles and in cardiac muscle, resulting in vessel relaxation and decreased heart rate and contractility.

2.2 Drug of study

2.2.1 Nifedipine (*Adalat LA*)

It is an extended release tablet dosage form of the calcium channel blocker.

It is a 3, 5-pyridinedicarboxylic acid, 1, 4-dihydro-2, 6-dimethyl-4-(2-nitrophenyl)-dimethyl ester, $C_{17}H_{18}N_2O_6$, and has the structural formula as shown below in figure 1:

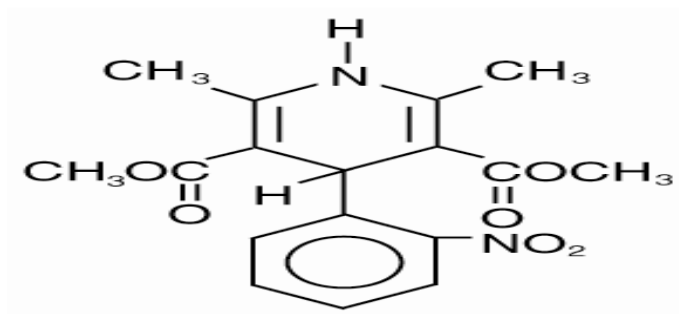


Figure 1: Diagram showing the structure of nifedipine- 3, 5-pyridinedicarboxylic acid, 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-dimethyl ester, [C₁₇H₁₈N₂O₆] (0893 5206 CCDS 1 Reference ID: 2 910908)

2.2.2 Mechanism of Action

Nifedipine's mechanism of action involves its ability to selectively inhibit calcium ion influx across the cell membrane, especially in the vascular smooth muscle and the heart muscle. The serum calcium ion concentrations remain stable, and the arterial blood pressure is lowered, causing a reduction in the peripheral vascular resistance and arterial vasodilation. Since nifedipine has the ability to prevent the influx of calcium ions into the cells, this will result in a reduction in peripheral vascular resistance with attendant lower arterial blood pressure and arterial vasodilation.

2.3 ORGAN OF STUDY

2.3.1 Ovaries

2.3.1.1 Gross Anatomy of the Ovary

Ovaries are the paired primary sex organs of the female that produce *gametes*, or *ova*, and the sex hormones, *oestrogens* and *progesterone*. In a sexually - matured female, these are solid, ovoid structures; and with each measuring about 3.5 cm (1.4 in.) long, 2 cm (0.8 in.) wide, and 1 cm (0.4 in.) thick – see figure 2A below (Van de Graff, 2001).

In rats, the ovaries are also paired and lie in the abdominal cavity, ventrolateral to the kidneys. Each ovary is small and, when matured, has a blistered appearance (Rowett, 1960).

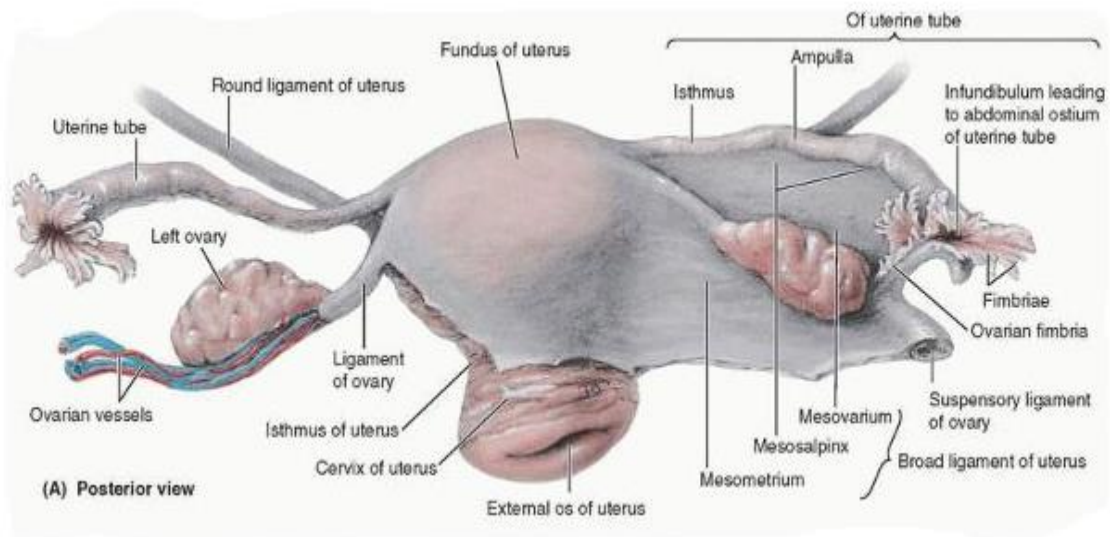


Figure 2A: Diagram showing an isolated dissection specimen consisting of the ovaries, uterine tubes, uterus, and related structures. (Source: Van de Graff, 2003).

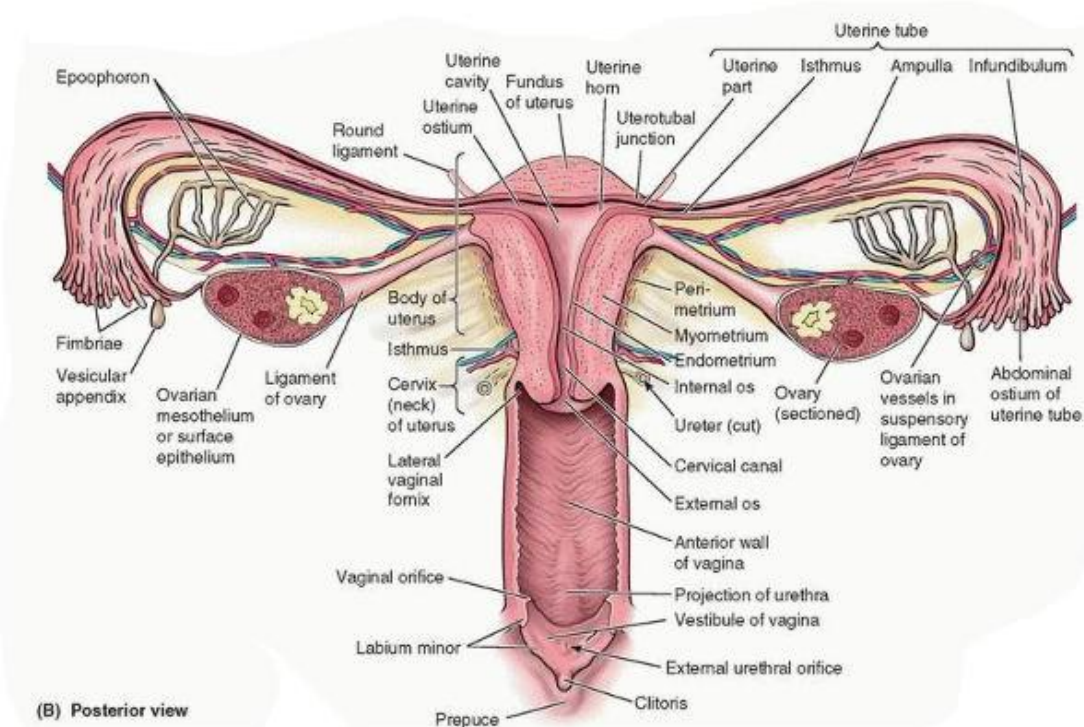


Figure 2B. Diagram showing the coronal section of the internal structure of the female genital organs. (Source: Van de Graff, 2003).

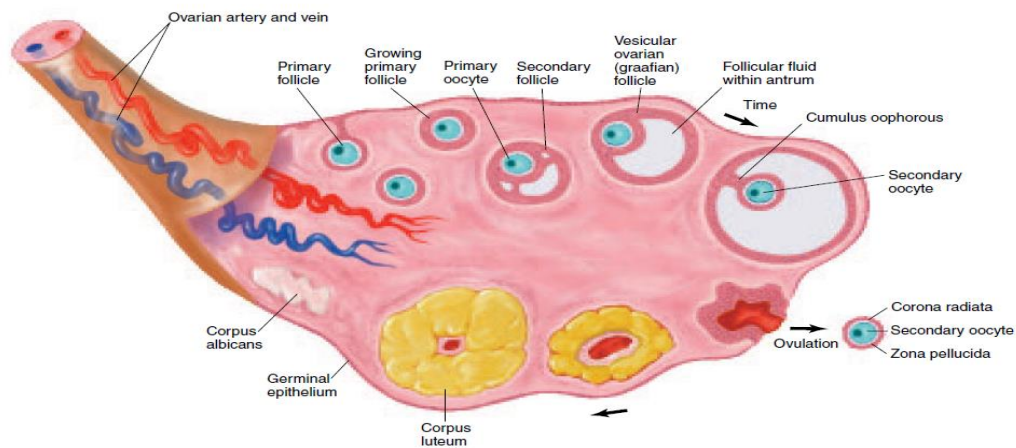


Figure 3: A schematic diagram of an ovary showing the various stages of ovum and follicle development. The arrows indicate changes with time.(Source: Van de Graff, 2003).

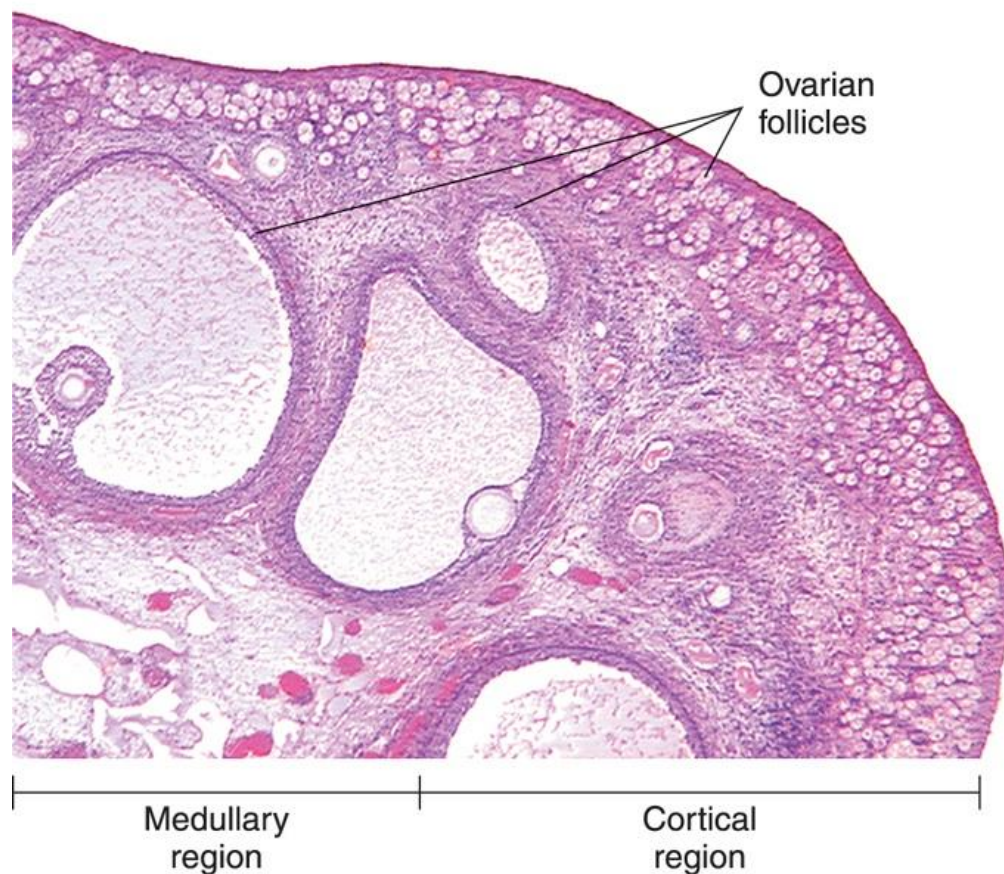


Figure 4: Photomicrograph of part of an ovary showing the cortical and medullary regions. H&E stain. Low magnification.(Junquiera & Carneiro, 2005)

2.3.2 Histology of the ovary (Figure 4)

The surface of the ovary is covered by a simple squamous or cuboidal epithelium called the germinal epithelium. Under this germinal epithelium is a layer of dense connective tissue called the tunica albuginea. This is responsible for the whitish colour of the ovary. Underneath the tunica albuginea is the cortical region wherein lie the ovarian follicles, containing predominately the oocytes. The follicles are embedded in the connective tissue (stroma) of the cortical region – see figure 4 above (Van de Graff, 2001). This stroma is composed of characteristic spindle-shaped fibroblasts that respond to hormonal stimuli in a different way than do fibroblasts of other organs.

In the innermost part of the ovary is the medullary region, containing a rich vascular bed within a loose connective tissue. There are no sharp limits (or demarcation) between the cortical and medullary regions – see figure 4 above (Van de Graff, 2001).

CHAPTER THREE

MATERIALS AND METHODS

3.1.0 MATERIALS:

3.1.1 Choice of antihypertensive: Adalat LA60

Adalat tablets, 60mg size (x30)

Manufactured by Bayer Schering Pharma AG, 51368 Leverkusen, Germany,

Nigeria. Ref. No: NAFDAC REG. NO.: A4 - 2373), Manufacturing date: 10/11/2010, Expiry date: 10/11/2014, Batch no: BXRT6JI.

3.1.2 Choice of animals: Wistar rats are the commonest, most friendly and most adaptive for animal experiment in this environment. They share similar morphological characteristics as humans.

The ethical approval for the study was obtained from the University / College's Committee on Ethics of Research.

3.1.3 Conflict of interest: None (This study did not and has not attracted any funding from any drug manufacturer). The result or outcome of this study will not be influenced by any organization or manufacturer; and part of the findings had been presented as an abstract at the Anatomical Society of Nigeria (ASN) Conference of July, 2014. Abstracts of some of the findings had also been sent to Science |Association of Nigeria (SAN) Conference in Lagos 2018 and the recently concluded MeDSABAMS / ASMeDA Conference in Abuja, 2018. The results of the study will be published in reputable peer – reviewed journals.

3.2.0 METHODS

The ethical approval for the study was obtained from the University / College's Committee on Ethics of Research, presumably with the approval of the proposal for the study.

3.2.1 Breeding of Animals:

Pure- bred Wistar rats were produced at 1st filial generation (F₁) level. Initially, 9 adult rats were acquired, consisting of 3 males to 6 females. These were acquired at the weaning stage from the Department of Biochemistry, University of Ilorin, Ilorin.

Animals were kept in wired cages in the College of Health Sciences' Animal House with the following conditions:

(a) prevalent temperature range 28⁰ to 31⁰C (28 ± 3°C) with photoperiodicity of 12: 12 light: darkness; and

(b) Six (6) female and three (3) male Wistar rats (*Rattus novergicus*) of about 150g were initially acquired and kept for them to mate so as to get first filial generation [F₁] of pure - bred, which were used in carrying out this research. The cages provided were wooden, demarcated and covered with a thick wire net and supplied with containers for water and feed. The animals were fed on pelletized growers feeds from Oluwanisola Top Feeds Ltd., Ajase-Ipo Road, Ilorin, containing crude protein (15%), fat (7%), crude fibre (10%), calcium (1%), phosphorus (0.35%), 2,550 Kcal /Kg as stated in the pack. Clean drinking water was provided to be taken *ad libitum*. A hygienic environment was ensured throughout the study. They were allowed to acclimatize for 2 weeks after which vaginal smearing was carried out to record the oestrus cycle of the female rats. After the 4th week, the animals were mated. The first filial pups thus produced were used for this study.

Thirty-six adult female Wistar rats weighing averagely 201.4 gm. (148.0 to 288.0 gm.) were used for this study, which was carried out in two stages, viz: non-pregnant and pregnant. They were put in different cages or compartment, with six animals in each cage.

3.2.2 Determination of the Oestrous Cycle Phase

The reproductive cycle of rats is called the oestrous cycle and can be divided into four different phases namely the prooestrus, oestrus, metoestrus and dioestrus (Long *et al.*, 1922). Ovulation commenced at the start of prooestrus phase and ends when oestrus phase is completed. The onset of sexual maturity in rats is calculated to be up to 12 months in age and the mean cycle length is around 4 to 5 days (Spornitz *et al.*, 1999).

The procedure involved introducing 0.3 to 0.5 ml of normal saline into the vagina with the aid of a Pasteur's pipette; and immediately the fluid was withdrawn, a drop of the fluid was then placed on a clean glass slide. A clean glass cover slip was placed on the drop, and the smear observed using the x 10 objective lens, with low illumination in the microscope and in the absence of the condenser lens to ensure a good contrast (Marcondes *et al.*, 2002). The four oestrus phases were thus identified and recorded. Further action depended on the stage of the study, pregnant or non- pregnant. The vaginal smear secretions were collected and viewed within 1-2 hours.

3.2.3 Vaginal Smear Cytology

The vaginal smear cytology was used to determine the oestrus cycle in rats. They were characterized by the proportion of the three types of cell observed in each of the phases. A prooestrus smear consists, predominantly, of round nucleated epithelial cells (Marcondes *et al.* 2002). Early in the morning between 8:00am and 9:00am, all the female rats were carried in plastic baskets to the research laboratory. Collections of vaginal secretions were done using plastic dropping pipettes (Pasteur's), each filled with about 0.3ml to 0.5ml of normal saline. By inserting the tip of the dropping pipette into rats' vagina and the bulb of the dropping pipette was pressurized, so as to allow the normal saline go into the vagina, and released so that the vaginal secretion goes back into the dropping pipette as shown in figure 5

(below). The collected secretions were placed on slides using at least one slide per rat. The slides were labelled with paper tape attached to one end.



Figure 5: Picture showing ways of holding the female rats for the collection of the vaginal lavage. (Marcondes, 1998)

The unstained vaginal smears were examined on glass slides under light microscope setting the magnification at x10 objective without using the condenser. The smear readings were recorded as shown in **Appendix I**. The prooestrus smear demonstrated anucleated cornified cells while the metoestrus slides revealed anucleated cells which are similar to the leukocytes in sizes. The dioestrous wet slides shows cells that are primarily secretory in nature and contains debris cellular substances with predominance of leukocytes. However, at the oestrus phase, the entire cells consist of anucleated, keratinised superficial cells that appear singly with scattered intermediary cells with intact nuclei.

After the vaginal smear had been carried out, the rats found at their prooestrus and oestrus phases were mated. They were also periodically weighed, in a medium-sized beaker, with an electronic weighing balance.

3.2.4 Mating of the rats and confirmation of mating

As soon as the ovulation period had been determined in the female rats, ‘hand breeding method’ was used in introducing a male to the females on the day it was noticed to be on heat (prooestrus). The pair was allowed to be together for about 14 hours (6pm to 8am). This is because rats mate more during the night than during the day.

Successful mating was confirmed by the presence of spermatozoa after carrying out a vaginal smear the following morning, and viewing under the light microscope (Marcondes *et al.*, 2002). This was taken to mark the beginning of the first day of conception (gestational day 1).

Assumed pregnant rats were isolated immediately and placed in appropriate group, as earlier determined, and treatment commenced immediately.

3.2.5 Preparation of Nifedipine Solution and Dosage

Five (5) tablets of Adalat LA [Nifedipine] tablets, each weighing 60mg, were slightly crushed and dissolved in distilled water for about 4 days to achieve total dissolution.

Calculation of dosage: since the therapeutic use of Nifedipine is between 20mg to 90mg and since normal physiological man weighs 70kg, therefore: $\frac{20mg}{70kg} = 0.3mgkg^{-1}$ body weight, therefore the minimal dose given in human is 0.3mg/kg body weight. However, a higher minimal dose of 1.5mg kg^{-1} body weight was given to the rats in this study because of the relatively very high metabolic rate in these animals; while the animals in the second experimental group were each given double dose (i.e. 2 X 0.3mg/kg. body weight =

3.0 $mgkg^{-1}$ body weight) daily. 0.1% solution of the drug was prepared for administration to the treated groups.

3.2.6 Treatment of Animals: The following tables (Table 1a & b) clearly show how the animals were grouped in the two stages of the study. However, while the eighteen animals in the non-pregnant stage were treated simultaneously from first day to the 21st day, those in the pregnant were serially treated after grouping. Each rat in the latter group was treated and followed through for 21 days after confirmation of mating.

3.2.6.1 Experimental Design Protocol for Non-Pregnant Rats

Table 1a: Table showing Experimental Design Protocol for Non-Pregnant Wistar Rats
(details of animal grouping and treatment in the group)

Group	Number of Rats (n)	Daily treatment	Route of Admin.	Duration (Days)
Group I (Control)	6	10.0 ml Kg^{-1} body weight Distilled Water	Orogastric	21
Group II (low dose)	6	1.5mg Kg^{-1} body weight Adalat LA soln	Orogastric	21
Group III (high dose)	6	3.0mg Kg^{-1} body weight Adalat LA soln	Orogastric	21

3.2.6.2 Experimental Design Protocol for Pregnant Rats

Table 1b: Table showing Experimental Design Protocol for Pregnant Wistar Rats (details of animal grouping and treatment in the group)

Group	Number of Rats (n)	Daily treatment	Route of Admin.	Duration (Days)
Group I (Control)	6	10.0 ml Kg^{-1} body weight Distilled Water	Orogastric	21
Group II (low dose)	6	1.5mg Kg^{-1} body weight Adalat LA soln	Orogastric	21
Group III (high dose)	6	3.0mg Kg^{-1} body weight Adalat LA soln	Orogastric	21

Animals were selected based on cycling and successful mating with a male partner, as shown by presence of spermatozoa in the vaginal smear the following morning.

3.2.6.3 Euthanasia, Sacrifice and Tissue Perfusion Fixation

At the end of the day 21 of treatment, the animals were anaesthetized by giving each rat ketamine hydrochloride 25mg intraperitoneally (IP). Thoracolaparotomy was performed to open the thorax and the abdomen, blood was taken from the heart through the right ventricle. Total body perfusion was done by infusing normal saline via the left ventricle, firstly to remove the blood from vascular spaces, followed by the introduction of 4% paraformaldehyde for general body fixation (Sprando's technique) (see Appendix VII). This was then followed by the removal of both ovaries. In the pregnant groups, the foetus-containing uteri were also harvested and preserved for subsequent morphological assessment of the products of conception (i.e. pups, cords and the placentae). The protocol for preparation of the fixatives was as outlined in Appendix II.

Blood samples, collected from each animal using a calibrated syringe and needle, were immediately put into sample bottles [Ethelene Diamine Tetraacetic Acid (EDTA) - and Lithium chloride – laden] for haematological tests and hormonal assay. The ovaries harvested were further immersed into 4% paraformaldehyde (PFA) solution already poured in the specimen bottles. The ovaries were trimmed of fat and dehydrated in increasing concentrations of ethanol (70%, 90% and two tranches of Absolute Ethanol) and thereafter embedded in paraffin wax. Serial sections of 5 µm thick were made. These were later stained with haematoxylin and eosin in the routine H & E preparation (Pearse, 1980). In addition, the ovarian sections from the pregnant rats were further stained with Masson's trichrome (MT) for the stroma, Fuelgen DNA (F-DNA) and immunohistochemical staining for oestrogen and progesterone receptors (GemRook Diagnostic Services).

3.2.7 Haematological Analysis Procedure

The full blood count parameters done included, packed cell volume (PCV), haemoglobin estimation, red blood cell (RBC) count, white blood cell (WBC) count, platelet count and blood film morphology. The latter included white cell differential counts for the proportions of the types of white blood cells. The blood samples collected from the animals were stored in EDTA-laden bottles from which they were collected in time to run the analysis.

3.2.8 Hormonal Studies:

Blood, collected in both experimental and control rats with plain bottles, once clotted, was further centrifuged at 3000 rpm for 15 minutes. The upper supernatant was removed off the specimen bottle. The sera, thus obtained, were transferred to other plain bottles for hormonal assay. The sera were kept frozen at -20⁰ C for hormonal assay. Serum levels of FSH, LH, Oestradiol and Progesterone were assayed. The reagents used and the procedures were as stated in **Appendix VI**.

3.2.9 Other procedures: The uterii were opened to deliver the pups. These were counted, weighed, superficially examined for any morphological anomalies and documented. The placentae were also inspected to check for any abnormalities. The pups were not dissected.

3.2.10 Tissue Processing for Paraformaldehyde-fixed Tissues

Tissue processing was done using facilities available at the Department of Histopathology, Faculty of Veterinary Medicine, University of Ilorin, Ilorin. Automatic tissue processor (Leica TP1020, Leica Biosystems ^R,Germany) was used, and the protocol was as stated in **Appendix III**. The tissues were embedded in paraffin wax using Tissue Embedding System (Leica TP1160, Leica Biosystems ^R,Germany). Tissue blocks were sectioned using a rotary microtome (Leitz Wetzlar ^R1512, Germany) at a thickness of 5µm. Sections were floated on glass slides placed on Slide Warming Table (Yorco ^R YSI 162, Yorco Scientific Industries Pvt Ltd., India), at about 40⁰C, and excess water drained from the sides.

3.2.11 Histological and Immunohistochemical Techniques

The following staining techniques were carried out using the facilities available at the Department of Morbid Anatomy, University of Ilorin Teaching Hospital [U.I.T.H.], Ilorin.

The reagents used and the procedures were as stated in **Appendices IV & V**.

3.2.12 Haematoxylin and Eosin Staining Technique (Pearse, 1980)

This technique was meant to reveal the normal architecture and the general outline of the ovary. The reagents used and the procedures were as stated in **Appendix IV**.

3.2.13 Masson's Trichrome staining Technique (Crookham & Dapson, 1991)

Masson's trichrome is a three-colour staining protocol used in histology. The recipes evolved from Claude L. Pierre Masson's (1880-1959) original formulation have different specific applications, but all are suited for distinguishing cells from surrounding connective tissue.

Most recipes produce red keratin and muscle fibres, blue or green collagen and bone, light red or pink cytoplasm, and dark brown to black cell nuclei. The reagents used and the procedures were as stated in **Appendix VII**.

3.2.14 Light Microscopy

For viewing the histological and histochemical sections, a binocular light microscope [.....] was used. The objective lenses used were of x10, x16 and x40 magnifications. Meanwhile, a Motic Camera (Moticam 2300; 3.0M pixel USB 2.0; Motic^R, Hong Kong) was attached to the microscope, and then connected to a computer with installed Moticam Software for microscope image analysis.

3.2.15 Data Analysis

The results were analysed by student "t" test using a Statistical Package for the Social Sciences (SPSS) Software version 16.0 (Microsoft Inc.^R). Data were presented as Mean $\pm$ SEM with the determination of level of significance set at p values < 0.05.

3.2.16 Qualitative analysis

Histological slides prepared were observed under light microscope at objective pieces x10, x16 and x40. Photographs were taken using digital cameras, and lately using AmScope digital camera. A qualitative comparison was done between the control group and the treated groups.

CHAPTER FOUR

4.0 RESULTS

4.1.0 MORPHOLOGICAL

4.1.1 WEIGHTS OF THE NON-PREGNANT ANIMALS

Table 2: Showing mean body weights of non-pregnant rats in the control group before administration and before sacrifice.

Rats	BA(in Grams)	BS(in Grams)
F1	158	219
F6	154	215
F8	162	224
F9	165	222
F11	158	198
F18	160	237
Average	159.5	219.16

Key: (BA= before administration, BS=before sacrifice)

Table 3: Showing average mean body weights of non-pregnant rats in group II (low-dose nifedipine-treated) before administration and before sacrifice.

Rats	BA(in Grams)	BS(in Grams)
F2	152	169
F3	158	203
F10	165	226
F12	176	229
F14	198	182
F17	162	215
Average	168.5	204.0

Key: (BA= before administration, BS= before sacrifice)

Table 4: Showing average mean body weights of non-pregnant rats in group III (high-dose nifedipine-treated) before administration and before sacrifice.

Rats	BA(in Grams)	BS(in Grams)
F4	185	230
F5	164	198
F7	187	214
F13	184	205
F15	184	234
F10	156	224
Average	176.67	218.33

Key: (BA= before administration, BS=before sacrifice)

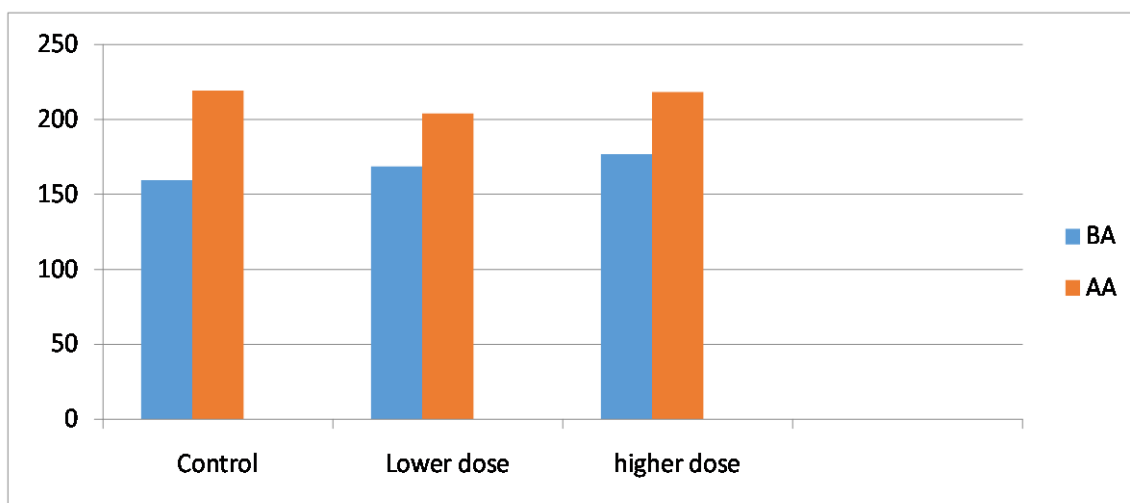
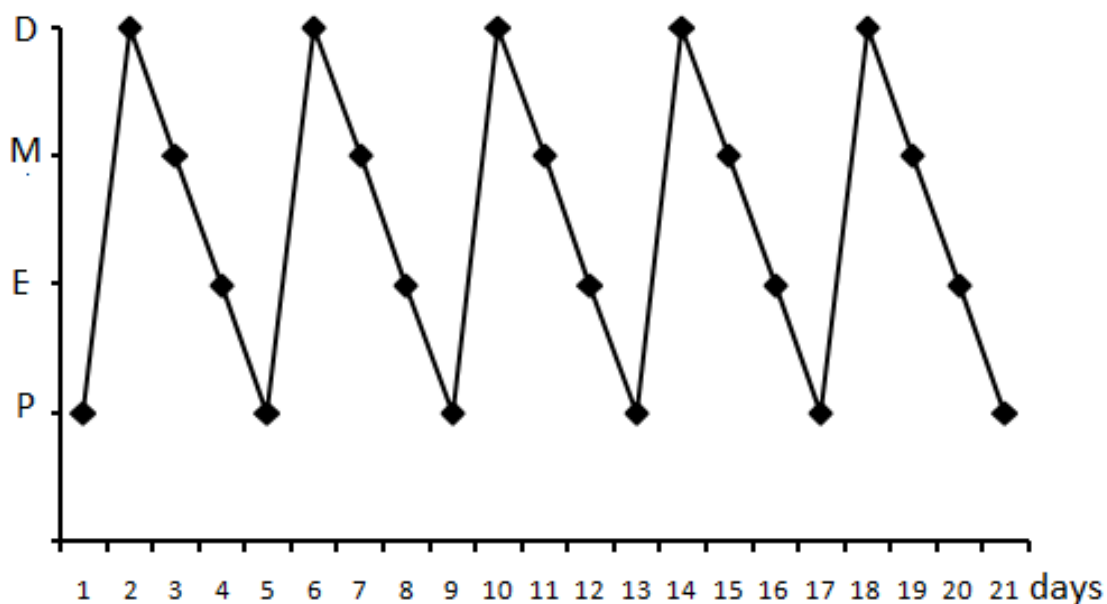


Chart 1: Bar chart showing the average body weights (in grams.) of all the non-pregnant rats before administration (BA) and before sacrifice (BS).

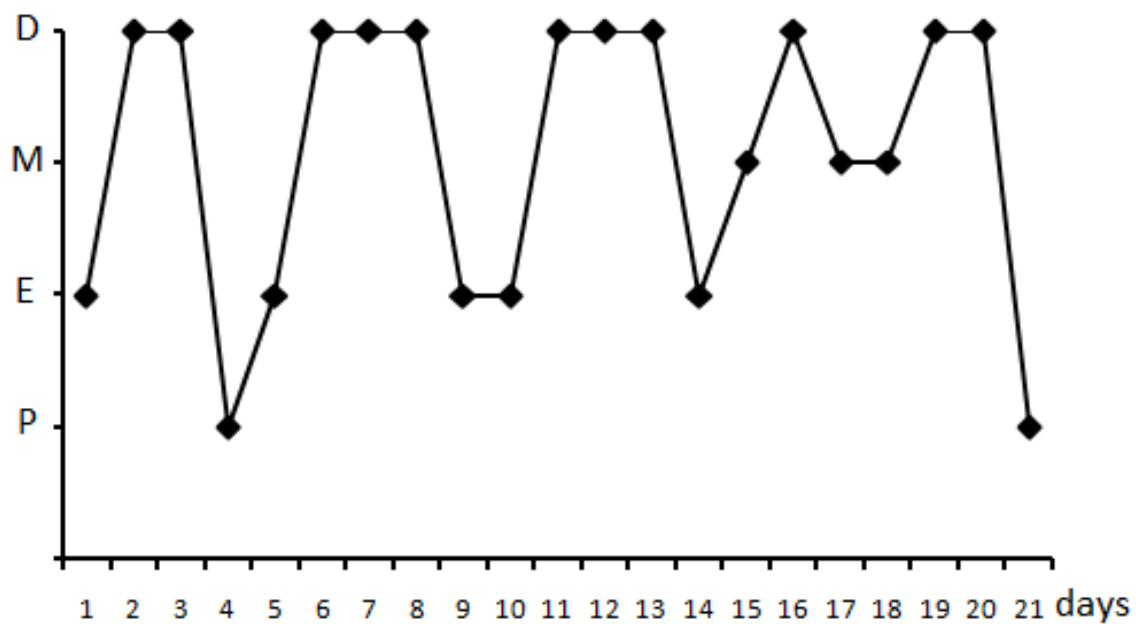
4.2.0 VAGINAL SMEAR READINGS

The readings are as shown in the tables below – see tables A, B & C in Appendix I. Analysis of the findings showed that the oestrus cycles in the rats in the control group ranged from 3 to 4 days with an average of 4 days. These parameters were respectively 1 to 12 days with an average of 5.13 days in rats administered 1.5 mg. of nifedipine per kg. body weight and 1 to 15 days with an average of 4.5 days in rats administered 3.0 mg. of nifedipine per kg. body weight as shown in the following graphs (Graphs 1 to 3 below)



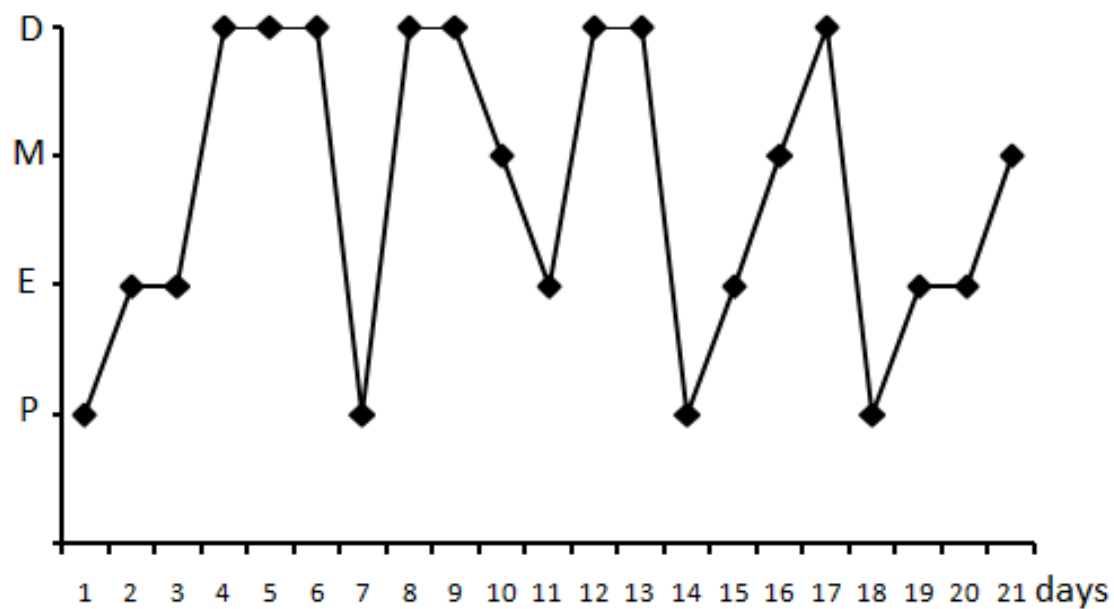
Graph 1: Showing the pattern of the vaginal smear readings (oestrus cycle) of the non-pregnant control rats.

Key: E- Oestrus, D- Dioestrus, M- Metoestrus, P- Prooestrus [Range 3 – 4days; Average duration = 4days]



Graph 2: Showing the pattern of the vaginal smear readings (oestrus cycle) in group II (low-dose nifedipine-treated) non-pregnant rats

Key: E- Oestrus, D- Dioestrus, M- Metoestrus, P- Prooestrus [Range 1 – 12days; Average duration = 5.13days]



Graph 3: Showing the pattern of the vaginal smear readings (oestrus cycle) in group III (high-dose nifedipine-treated) non-pregnant rats

Key: E- Oestrus, D- Dioestrus, M- Metoestrus, P- Prooestrus [Range 1 – 15days; Average duration = 4.5days]

4.3.0 HORMONAL ASSAY RESULTS

Serum progesterone levels were significantly increased in the treated non-pregnant groups (low- and high- dose nifedipine-treated groups (26.18 ± 2.33 and 27.2 ± 5.89 ng/ml.) compared with the control group (24.17 ± 0.96 ng/ml.); $p < 0.05$ (**See Table 5**).

In the pregnant rats, serum levels of progesterone were respectively 94.18 ± 0.96 , 98.43 ± 2.33 and 92.52 ± 5.89 ng/ml. in the control, low- and high- dose nifedipine-treated groups (**See Table 6**).

Mean serum oestradiol level in the non-pregnant control group was 41.00 ± 6.14 pg/ml. as against the low-dose 75.83 ± 12.61 pg/ml. and the high-dose nifedipine-treated, 34.67 ± 6.69 pg/ml. There were highly significant differences between these groups; $p < 0.05$ (**See Table 5**).

Similarly, the mean serum oestradiol level in the pregnant control group was 1750.00 ± 220.39 pg/ml. as against the levels in the low-dose (1975.00 ± 442.72 pg/ml.) and the high-dose nifedipine-treated, (1661.67 ± 290.96 pg/ml). These were relatively very high when compared with the levels in the non-pregnant animals (**See Table 6**).

The mean serum level of luteinizing hormone in the non-pregnant control group was 9.48 ± 1.45 mIU/ml. as against the corresponding value of 13.55 ± 5.51 mIU/ml. in the low-dose nifedipine-treated. There were increases in the serum LH concentration in the low-dose nifedipine-administered rats compared with those of the control group; $p < 0.05$ (**See Table 5**). In the high-dose nifedipine-treated, the mean serum level of luteinizing hormone was 7.36 ± 0.65 mIU/ml.; this was clearly lower!

The mean serum level of luteinizing hormone in the pregnant control group was 2.71 ± 0.26 mIU/ml. as against the corresponding value of 11.20 ± 9.21 mIU/ml. in the low-dose

nifedipine-treated. There were increases in the serum LH concentration in the low-dose nifedipine-administered rats compared with those of the control group; $p < 0.05$ (**See Table 6**). In the high-dose nifedipine-treated, the mean serum level of luteinizing hormone was 2.92 ± 0.24 mIU./ml.; this was also clearly lower!

However, there were no statistically significant differences between the serum levels of FSH across the non-pregnant experimental groups; these were respectively 2.72 ± 0.43 , 2.92 ± 0.51 and 2.73 ± 0.32 mIU./ml in the control, low-dose and high-dose nifedipine-treated groups (p value < 0.05 , **See Table 5**).

The mean serum level of FSH in the pregnant control group was 0.68 ± 0.33 mIU./ml. as against the corresponding value of 1.30 ± 0.95 mIU./ml. in the low-dose nifedipine-treated. The increases in the serum FSH concentration in the low-dose nifedipine-administered rats were almost 100% compared with those of the control group; $p < 0.05$ (**See Table 6**). In the high-dose nifedipine-treated pregnant rats, the mean serum level of FSH was 1.28 ± 0.45 mIU./ml.; this was also almost 100% of those in the control pregnant!

Table 5: Table showing hormonal parameters of non-pregnant rats in control group, low- and high-dose nifedipine-treated groups.

Parameters	Control	Low dose	High dose
Progesterone (ng/ml)	24.17 ± 0.96	26.18 ± 2.33	27.2 ± 5.89
Oestradiol (pg/ml)	41.00 ± 6.14	75.83 ± 12.61	34.67 ± 6.69
LH (mI.U./ml)	9.48 ± 1.45	13.55 ± 5.51	7.36 ± 0.65
FSH (mI.U. / ml)	2.72 ± 0.43	2.92 ± 0.51	2.73 ± 0.32

N.B.: Values are expressed as mean ± SEM; *p* value < 0.05

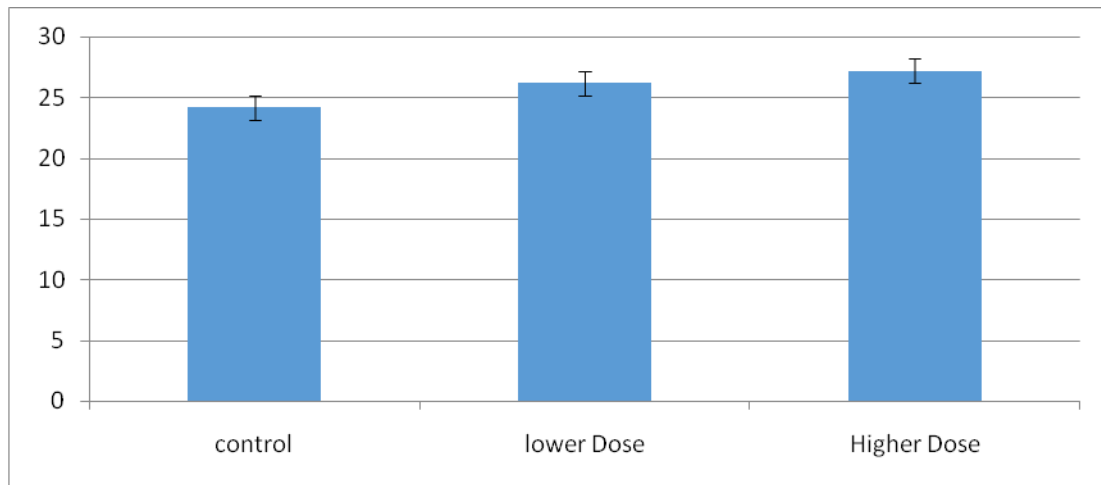


Chart 2: Bar chart showing serum progesterone levels, in nonagram per millilitre (ng/ml), in non-pregnant rats.

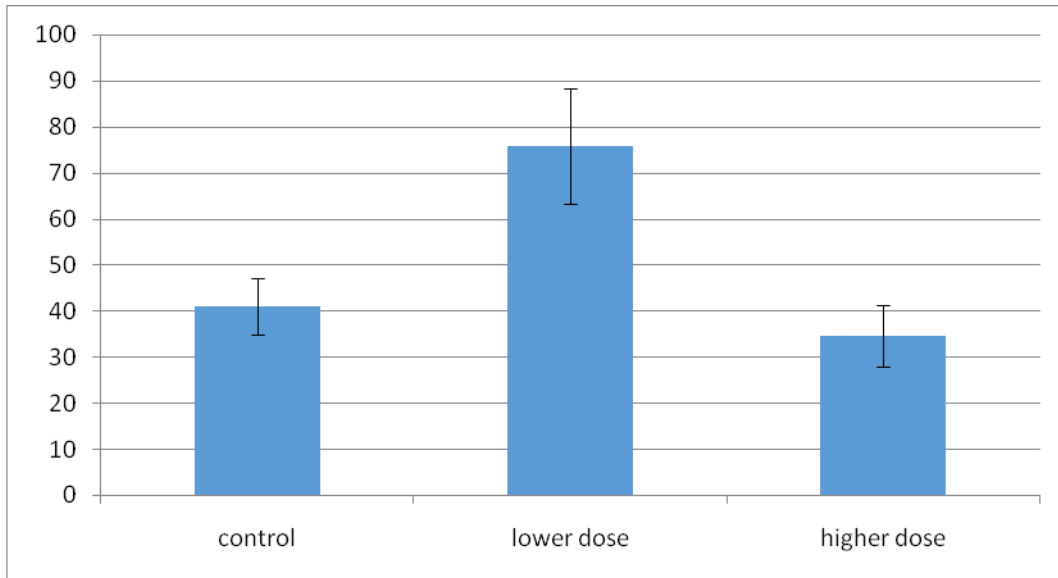


Chart 3: Bar chart showing serum oestradiol levels, in picogram per millilitre (pg/ml), in non-pregnant rats.

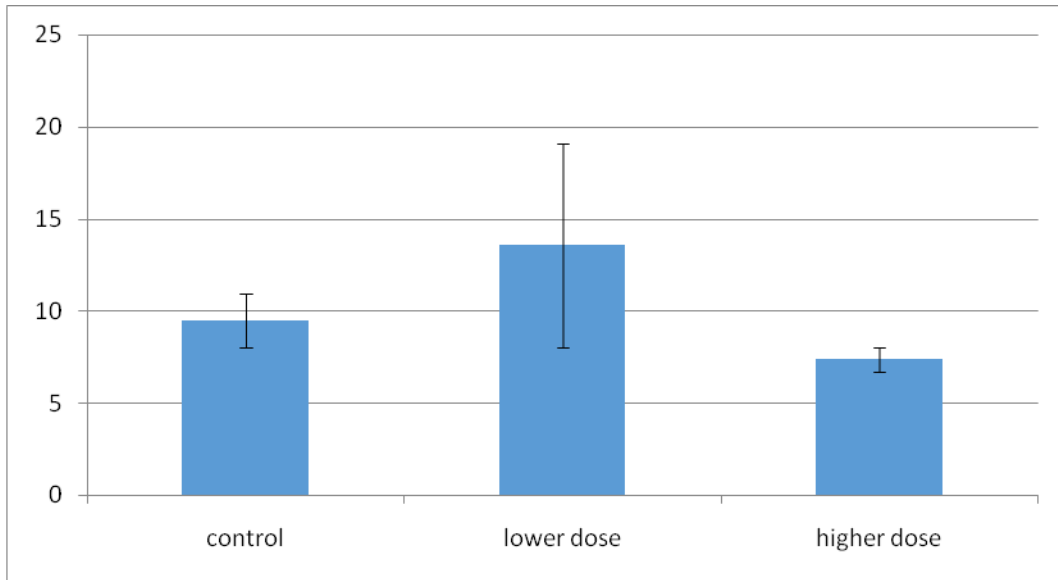


Chart 4: Bar chart showing serum luteinizing hormone (LH) levels, in milli-International Units per milliLitre (mIU/ml), in non-pregnant rats.

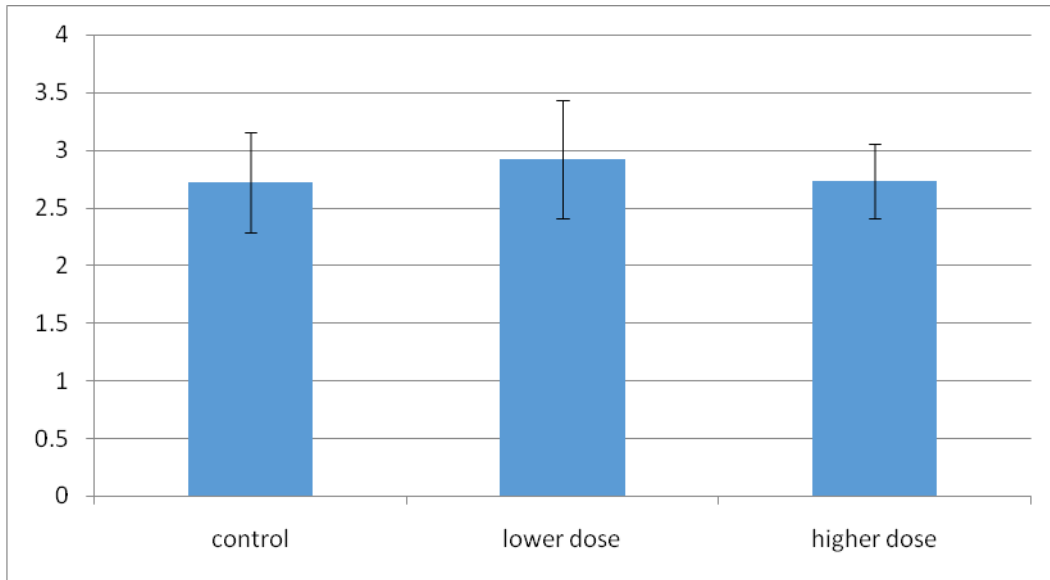


Chart 5: Bar chart showing serum follicle-stimulating hormone (FSH) levels, in milli-International Units per millilitre (mIU/ml), in non-pregnant rats.

Table 6: Showing hormonal parameters of pregnant rats in control group, low- and high- dose nifedipine-treated groups.

Parameters	Control	Low dose	High dose
Progesterone (ng/ml)	94.18 ± 0.96	98.43 ± 2.33	92.52 ± 5.89
Oestradiol (pg/ml)	1750.00 ± 220.39	1975.00 ± 442.72	1661.67 ± 290.96
LH (mL.U. / ml)	2.71 ± 0.26	11.20 ± 9.21	2.92 ± 0.24
FSH (mL.U. / ml)	0.68 ± 0.33	1.30 ± 0.95	1.28 ± 0.45
Testosterone (ng/ml)	6.53 ± 0.23	7.11 ± 0.13	6.38 ± 0.09
Prolactin (ng/ml)	1.53 ± 0.21	2.60 ± 1.41	2.20 ± 0.22

N.B.: Values are expressed as mean ± SEM

4.4.0 HISTOARCHITECTURE OF THE OVARY

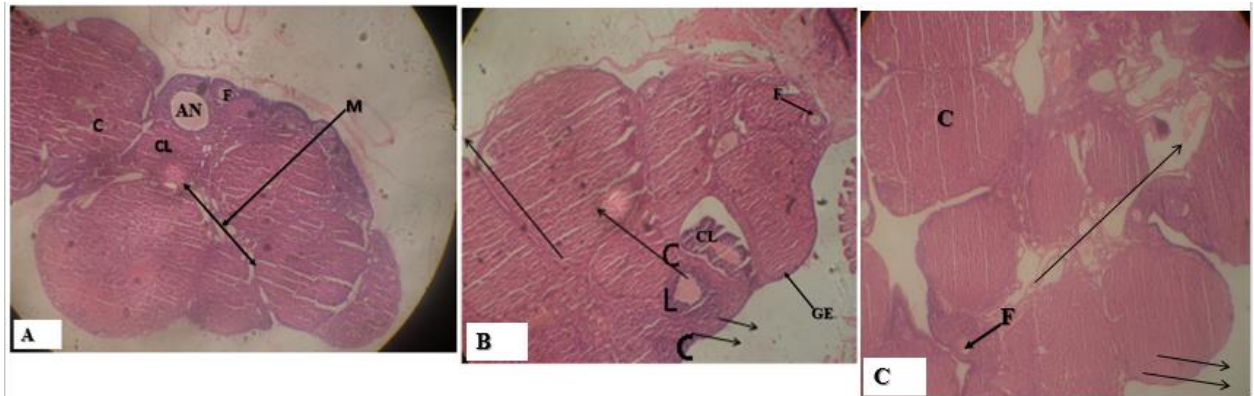


Plate 1: (A) Photomicrograph of ovary from a non-pregnant rat in the control group stained with Haematoxylin & Eosin Y (H & E)[Mag. x140.8] (B) Photomicrograph of ovary from a non-pregnant rat in the control group stained with Haematoxylin& Eosin Y (H & E)[Mag. = x 140.8] and (C) Photomicrograph of ovary from a non-pregnant rat in the high-dose nifedipine-treated group stained with Haematoxylin & Eosin Y (H & E)[Mag. = x 140.8].

Keys: M = Medulla; C = Cortex; CL = Corpus luteum; AN = follicular antrum; T= Theca layer; O = Oocyte; T = Theca layer; F = Primordial Follicle; GE = Germinal Epithelium.

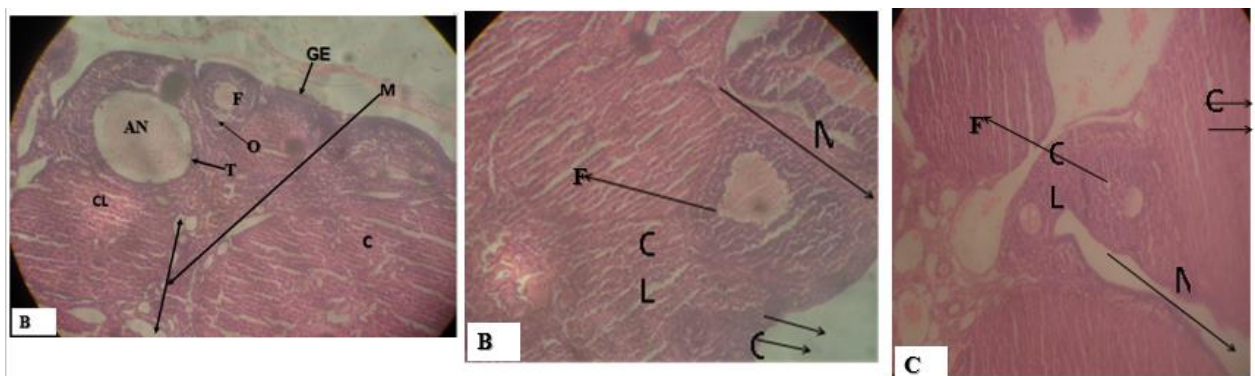


Plate 2: (A) Photomicrograph of ovary from a non-pregnant rat in the control group stained with Haematoxylin & Eosin Y (H & E)[Mag. X 352] (B) Photomicrograph of ovary from a non-pregnant rat in the control group stained with Haematoxylin & Eosin Y (H & E) [Mag. = X 352] and (C) Photomicrograph of ovary from a non-pregnant rat in the high-dose nifedipine-treated group stained with Haematoxylin & Eosin Y (H & E) [Mag. = X 352].

Keys: M = Medulla; C = Cortex; CL = Corpus luteum; AN = follicular antrum; T = Theca layer; O = Oocyte; T = Theca layer; F = Primordial Follicle; GE = Germinal Epithelium.

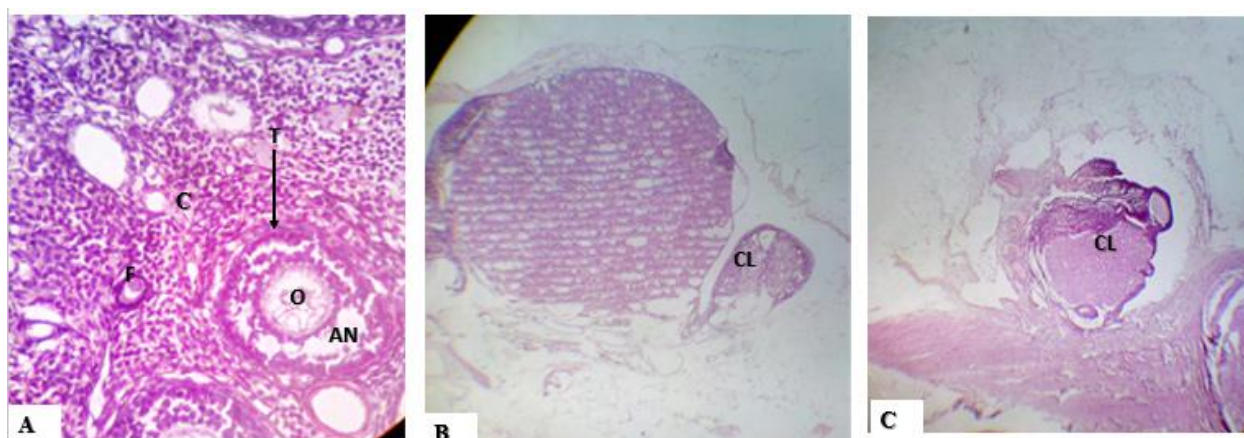


Plate 3: (A) Photomicrograph of ovary from a pregnant rat in the control group stained with Haematoxylin & Eosin Y (H & E)[Mag. = x 40]; (B) Photomicrograph of ovary from a pregnant rat in the low-dose nifedipine-treated group stained with Haematoxylin & Eosin Y (H & E)[Mag. = x 40] and (C) Photomicrograph of ovary from a pregnant rat in the high-dose nifedipine-treated group stained with Haematoxylin & Eosin Y (H & E)[Mag. = x 40].

Keys: C = Cortex; CL = Corpus luteum; AN = follicular antrum; T = Theca layer, O = Oocyte; T = Theca layer; F = Primordial Follicle.

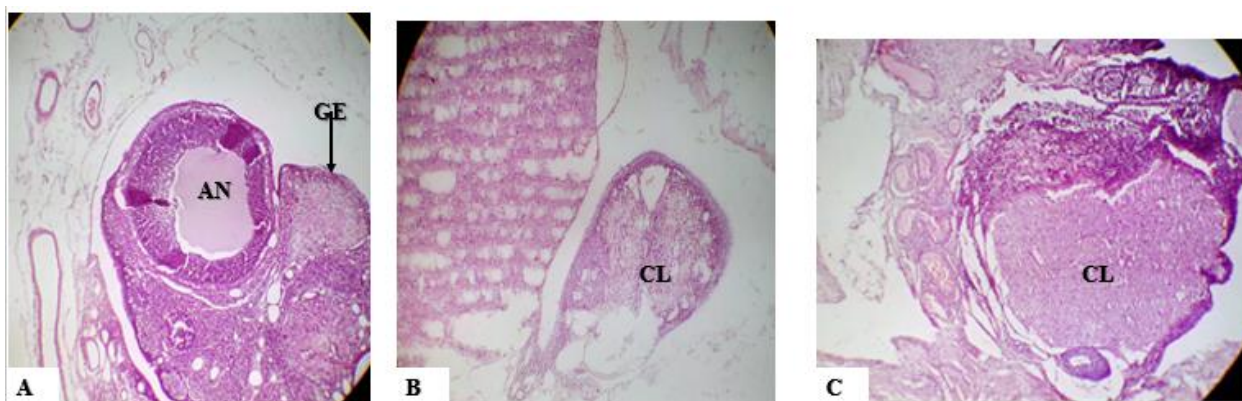


Plate 4: (A) Photomicrograph of ovary from a pregnant rat in the control group stained with Haematoxylin & Eosin Y (H & E)[Mag. = x 100]; (B) Photomicrograph of ovary from a pregnant rat in the low-dose nifedipine-treated group stained with Haematoxylin & Eosin Y (H & E)[Mag. = x 100] and (C) Photomicrograph of ovary from a pregnant rat in the high-dose nifedipine-treated group stained with Haematoxylin & Eosin Y (H & E)[Mag. = x 100].

Keys: CL = Corpus luteum; AN = follicular antrum; GE = Germinal Epithelium.

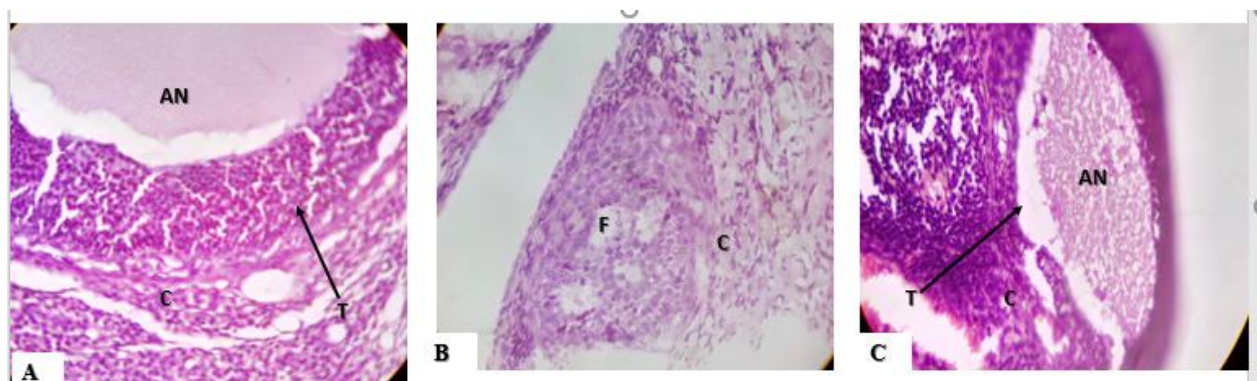


Plate 5: (A) Photomicrograph of ovary from a pregnant rat in the control group stained with Haematoxylin & Eosin Y (H & E)[Mag. = x400]; (B) Photomicrograph of ovary from a pregnant rat in the low-dose nifedipine-treated group stained with Haematoxylin & Eosin Y (H & E)[Mag. = x 400] and (C) Photomicrograph of ovary from a pregnant rat in the high-dose nifedipine-treated group stained with Haematoxylin & Eosin Y (H & E)[Mag. = x 400].

Keys: C = Cortex; CL = Corpus luteum; F = Follicle; AN = follicular antrum; T = Theca layer.

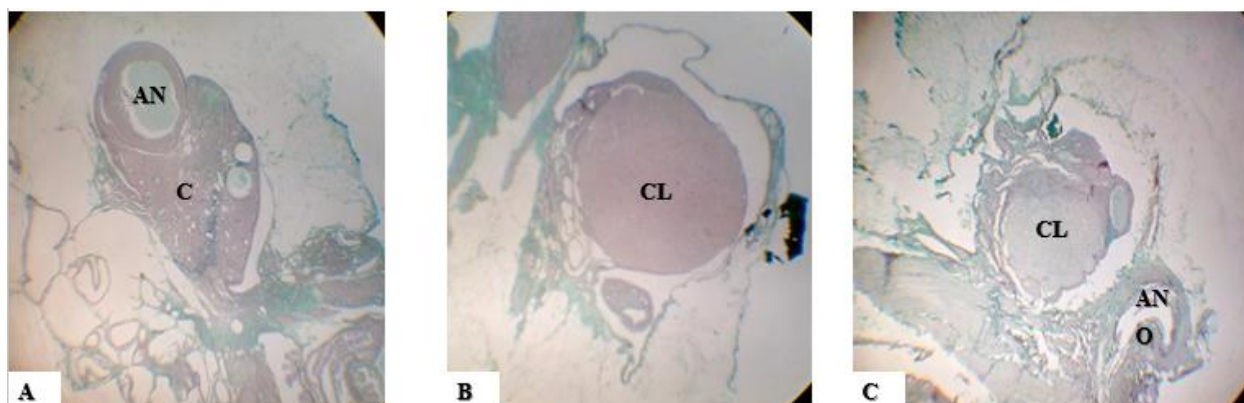


Plate 6: (A) Photomicrograph of ovary from a pregnant rat in the control group stained with Masson's Trichrome (MT)[Mag. = x 40]; (B) Photomicrograph of ovary from a pregnant rat in the low-dose nifedipine-treated group stained with Masson's Trichrome (MT)[Mag. = x 40] and (C) Photomicrograph of ovary from a pregnant rat in the high-dose nifedipine-treated group stained with Masson's Trichrome (MT) [Mag. = x 40].

Keys: C = Cortex, CL = Corpus luteum; AN = follicular antrum; O = Oocyte.

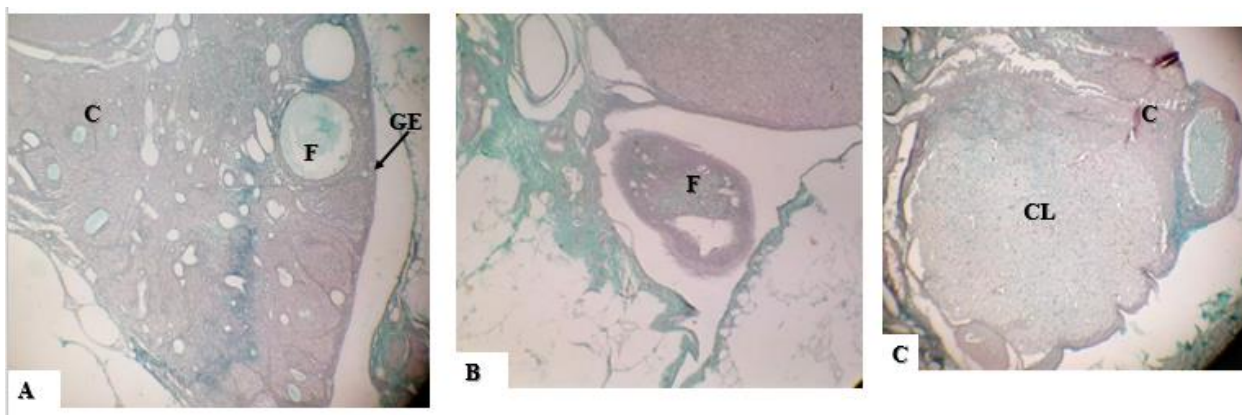


Plate 7: (A) Photomicrograph of ovary from a pregnant rat in the control group stained with Masson's Trichrome (MT)[Mag. = x 100]; (B) Photomicrograph of ovary from a pregnant rat in the low-dose nifedipine-treated group stained with Masson's Trichrome (MT)[Mag. = x 100] and (C) Photomicrograph of ovary from a pregnant rat in the high-dose nifedipine-treated group stained with Masson's Trichrome (MT) [Mag. = x 100].

Keys: C = Cortex; CL = Corpus luteum; F = Primordial Follicle; GE = Germinal Epithelium.

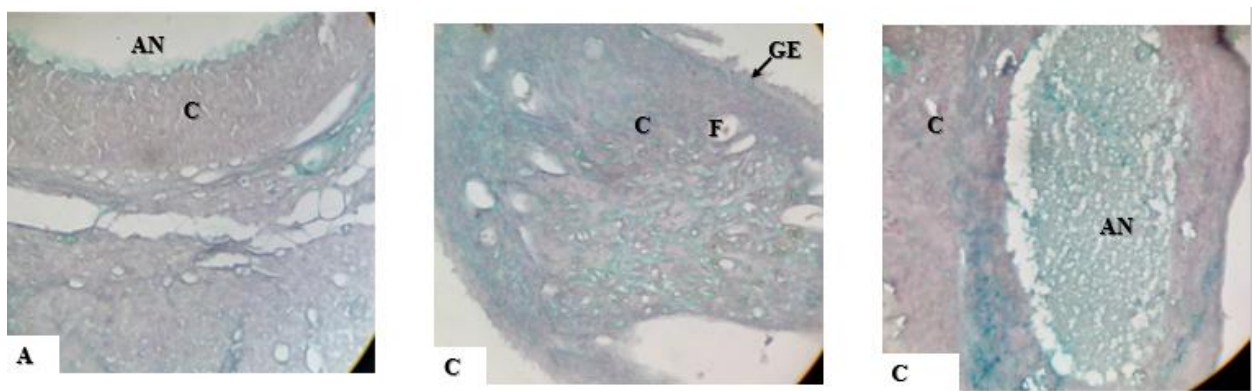


Plate 8: Photomicrograph of ovary from a pregnant rat (A) in the control group; (B) in the low-dose nifedipine-treated group and (C) in the high-dose nifedipine-treated group stained with Masson's Trichrome (MT) [Mag. = x 400].

Keys: C = Cortex; CL = Corpus luteum; AN = follicular antrum; F = Primordial Follicle; GE = Germinal Epithelium.

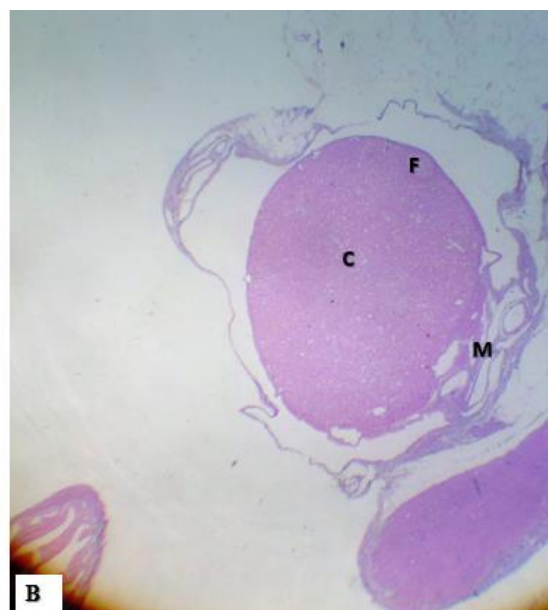
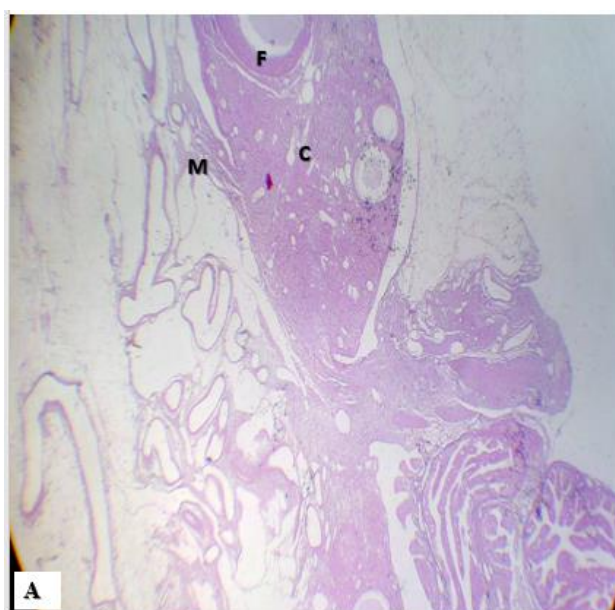


Plate 9: (A) Photomicrograph of ovary from a pregnant rat in the control group stained with Feulgen DNA (F-DNA)[Mag. = x 40] and (B) Photomicrograph of ovary from a pregnant rat in the low-dose nifedipine-treated group stained with Feulgen DNA (F-DNA) [Mag. = x 40].

Keys: M = Medulla; C = Cortex; CL = Corpus luteum; F = Primordial Follicle.

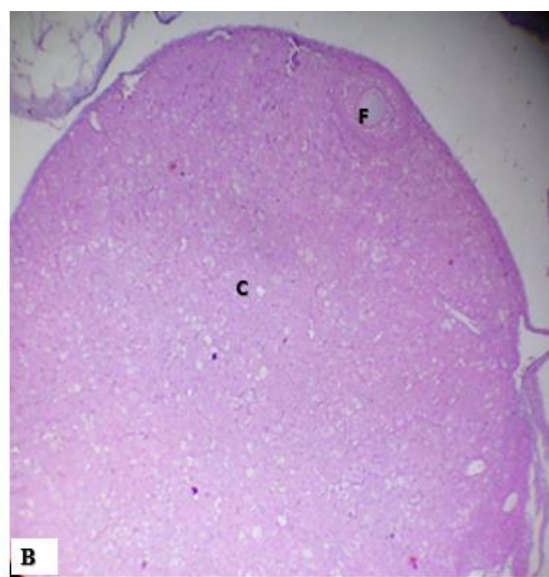
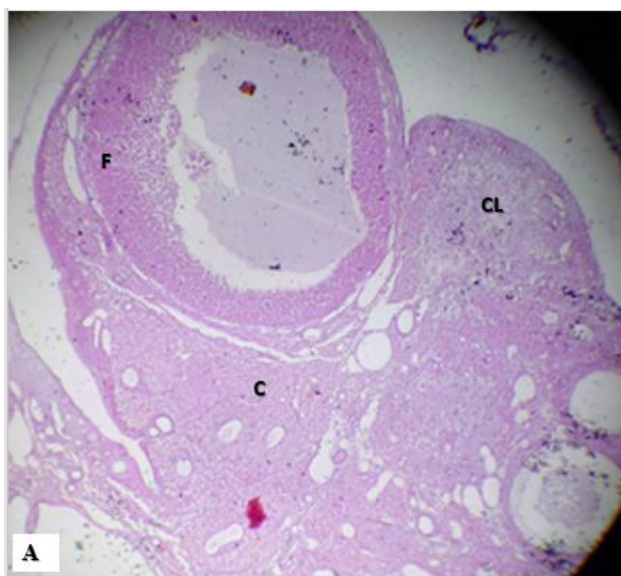


Plate 10: (A) Photomicrograph of ovary from a pregnant rat in the control group stained with Feulgen DNA (F-DNA)[Mag. = x100] and (B) Photomicrograph of ovary from a pregnant rat in the low-dose nifedipine-treated group stained with Feulgen DNA (F-DNA) [Mag. = x 100].

Keys: C = Cortex; CL = Corpus luteum; F = Primordial Follicle.

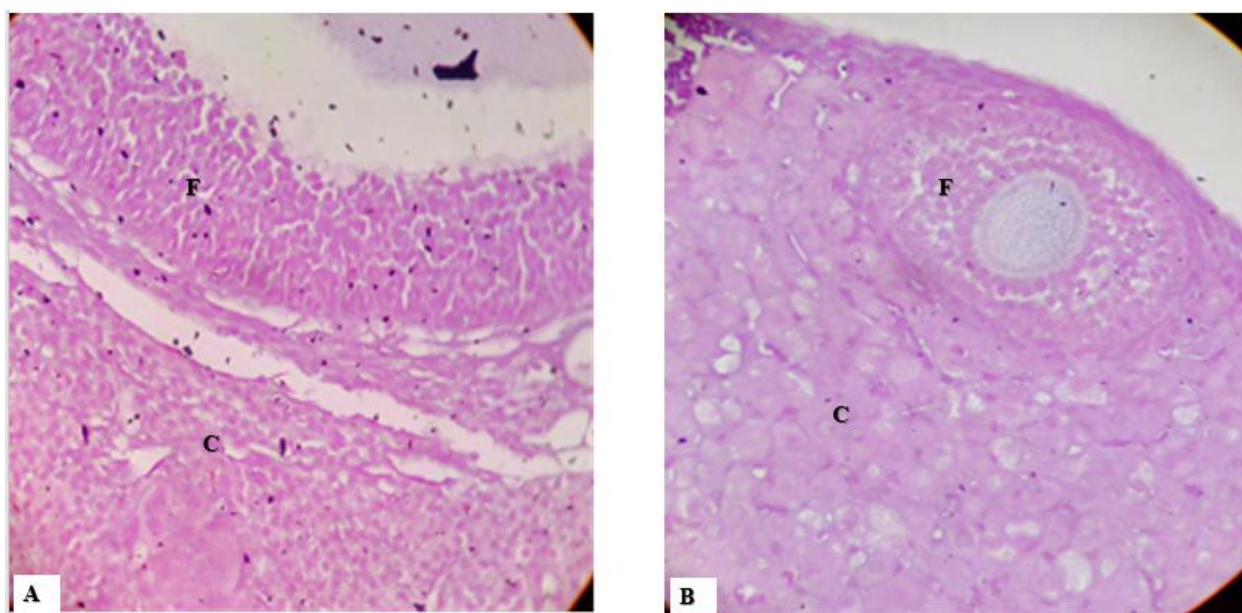


Plate 11: (A) Photomicrograph of ovary from a pregnant rat in the control group stained with Feulgen DNA (F-DNA)[Mag. = x400] and (B) Photomicrograph of ovary from a pregnant rat in the low-dose nifedipine-treated group stained with Feulgen DNA (F-DNA) [Mag. = x 400].

Keys: M = Medulla; C = Cortex; CL = Corpus luteum; F = Primordial Follicle.

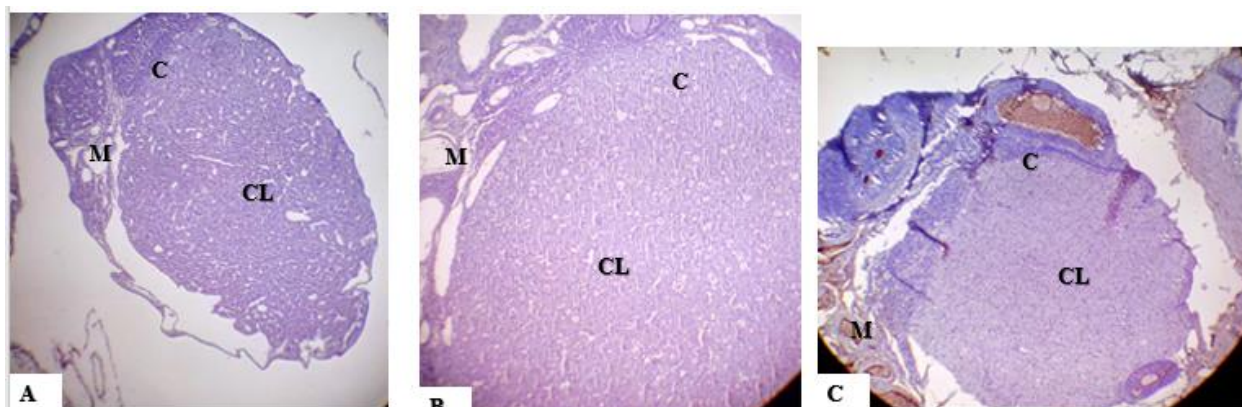


Plate 12: (A) Photomicrograph of ovary from a pregnant rat in the control group stained for Oestrogen Receptor (ER) [Mag. = x 100]; (B) Photomicrograph of ovary from a pregnant rat in the low-dose nifedipine-treated group stained for Oestrogen Receptor (ER) [Mag. = x 100] and (C) Photomicrograph of ovary from a pregnant rat in the high-dose nifedipine-treated group stained for Oestrogen Receptor (ER) [Mag. = x 100].

Keys: M = Medulla; C = Cortex; CL = Corpus luteum; F = Primordial Follicle; GE = Germinal Epithelium.

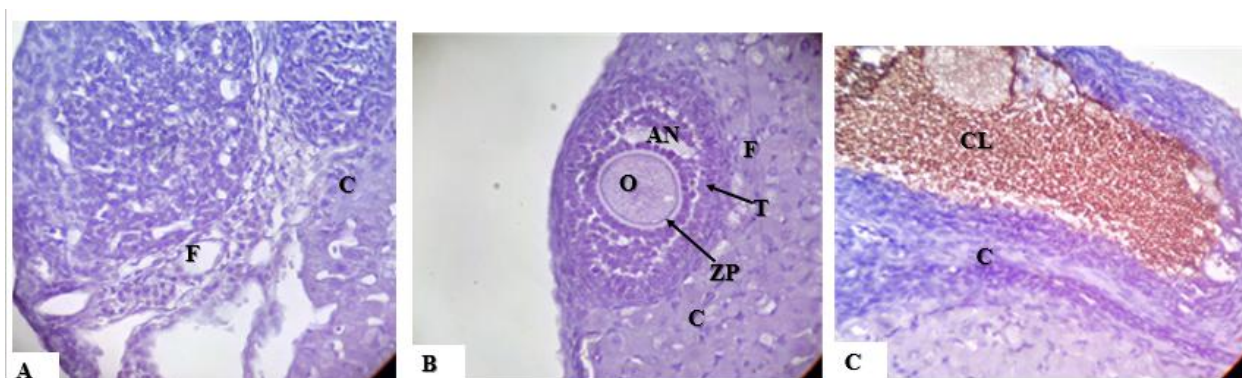


Plate 13: (A) Photomicrograph of ovary from a pregnant rat in the control group stained for Oestrogen Receptor (ER) [Mag. = x 400]; (B) Photomicrograph of ovary from a pregnant rat in the low-dose nifedipine-treated group stained for Oestrogen Receptor (ER) [Mag. = x 400] and (C) Photomicrograph of ovary from a pregnant rat in the high-dose nifedipine-treated group stained for Oestrogen Receptor (ER) [Mag. = x 400].

Keys: C = Cortex; CL = Corpus luteum; O = Oocyte; AN = Follicular Antrum; T = Theca layer; F = Primordial follicle; ZP = Zona pellucida.

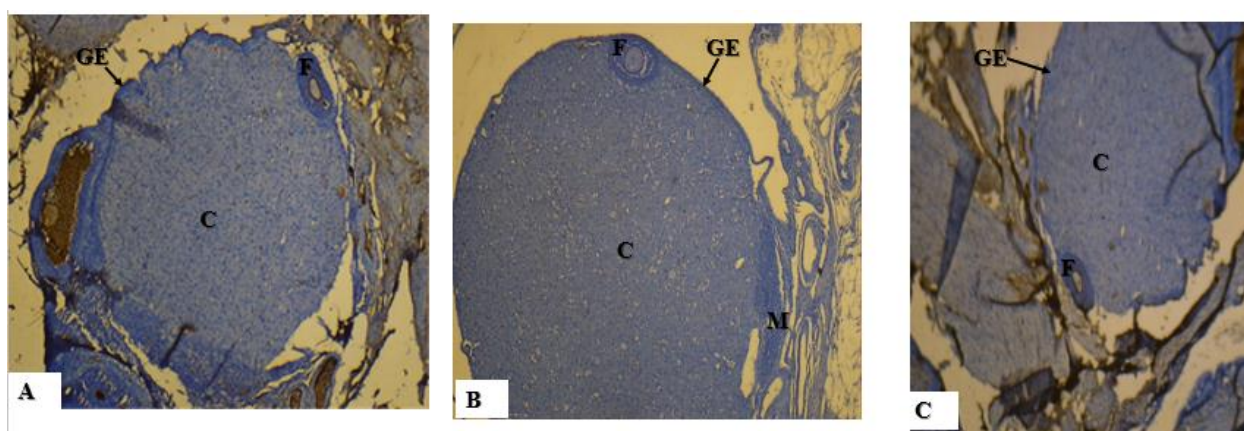


Plate 14: (A) Photomicrograph of ovary from a pregnant rat in the control group stained for Progesterone Receptor (PR) [Mag. = x 40]; (B) Photomicrograph of ovary from a pregnant rat in the low-dose nifedipine-treated group stained for Progesterone Receptor (PR) [Mag. = x 40] and (C) Photomicrograph of ovary from a pregnant rat in the high-dose nifedipine-treated group stained for Progesterone Receptor (PR) [Mag. = x 40].

Keys: M = Medulla; C = Cortex; F = Primordial Follicle; GE = Germinal Epithelium.

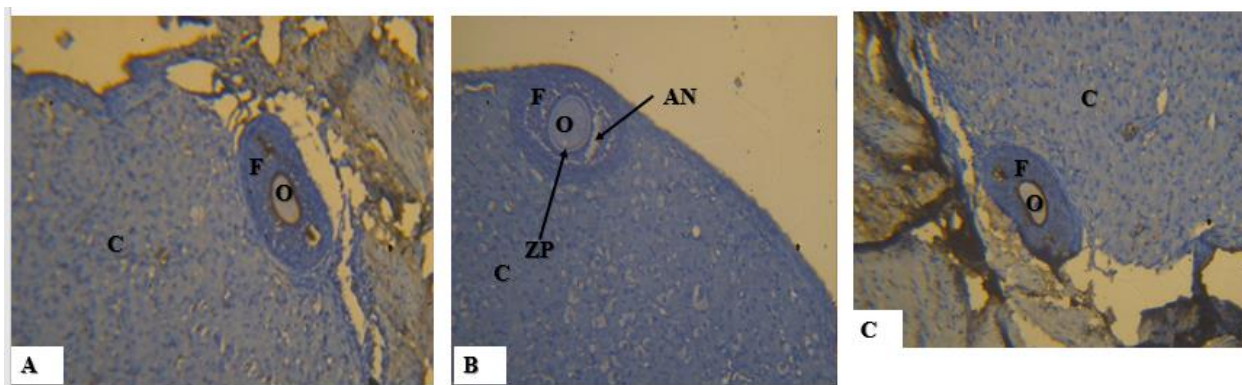


Plate 15 (A) Photomicrograph of ovary from a pregnant rat in the control group stained for Progesterone Receptor (PR) [Mag. = x 100]; **(B)** Photomicrograph of ovary from a pregnant rat in the low-dose nifedipine-treated group stained for Progesterone Receptor (PR) [Mag. = x 100] and **(C)** Photomicrograph of ovary from a pregnant rat in the high-dose nifedipine-treated group stained for Progesterone Receptor (PR) [Mag. = x 100].

Keys: C = Cortex; O = Oocyte; AN = Follicular Antrum; F = Follicle; ZP = Zona pellucida.

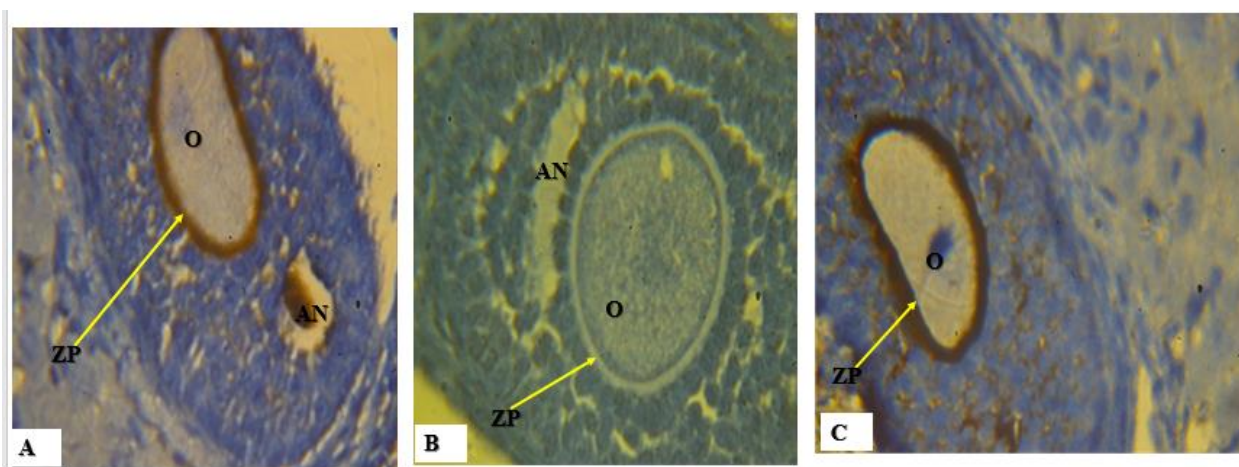


Plate 16: (A) Photomicrograph of ovary from a pregnant rat in the control group stained for Progesterone Receptor (PR) [Mag. = x 400]; (B) Photomicrograph of ovary from a pregnant rat in the low-dose nifedipine-treated group stained for Progesterone Receptor (PR) [Mag. = x 400] and (C) Photomicrograph of ovary from a pregnant rat in the high-dose nifedipine-treated group stained for Progesterone Receptor (PR) [Mag. = x 400].

Keys: O = Oocyte; AN = Follicular Antrum; F = Follicle; ZP = Zona pellucida.

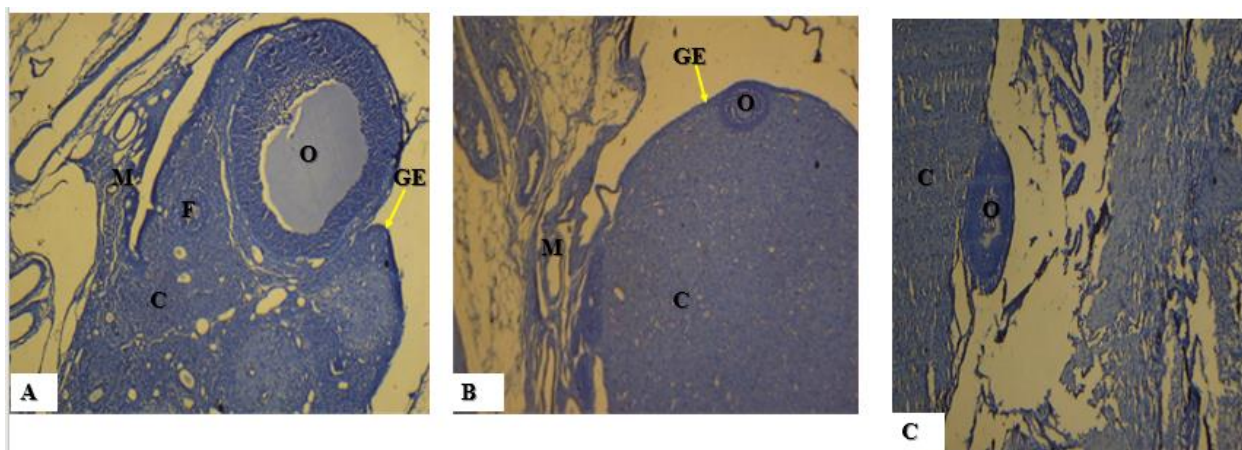


Plate 17: (A) Photomicrograph of ovary from a pregnant rat in the control group stained for Receptor (HER2) [Mag. = x 40]; (B) Photomicrograph of ovary from a pregnant rat in the low-dose nifedipine-treated group stained for Receptor (HER2) [Mag. = x 40] and (C) Photomicrograph of ovary from a pregnant rat in the high-dose nifedipine-treated group stained for Receptor (HER2) [Mag. = x 40].

Keys: M = Medulla; C = Cortex; O = Oocyte; F = Follicle; GE = Germinal Epithelium.

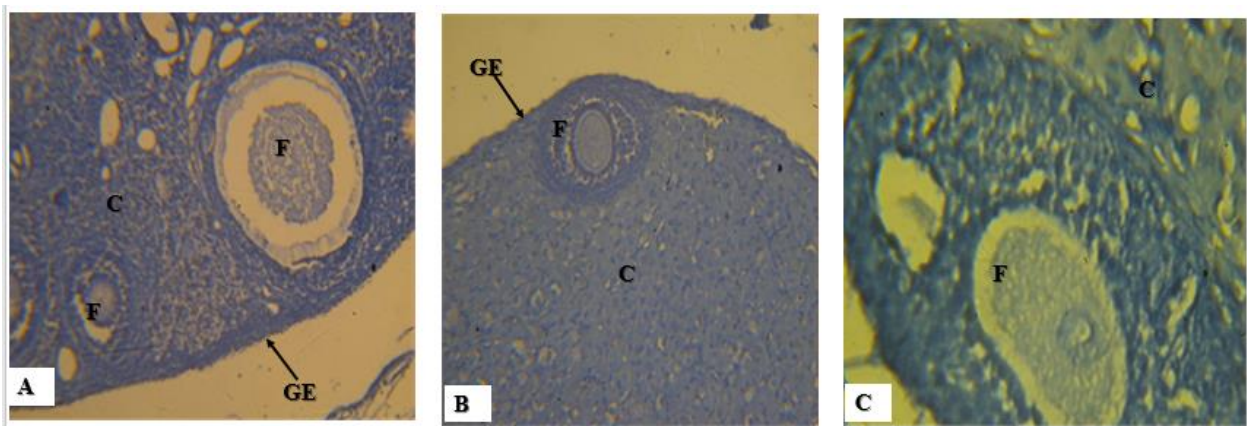


Plate 18: (A) Photomicrograph of ovary from a pregnant rat in the control group stained for Receptor (HER2) [Mag. = x 100]; (B) Photomicrograph of ovary from a pregnant rat in the low-dose nifedipine-treated group stained for Receptor (HER2) [Mag. = x 100] and (C) Photomicrograph of ovary from a pregnant rat in the high-dose nifedipine-treated group stained for Receptor (HER2) [Mag. = x 100].

Keys: C = Cortex; F = Follicle; GE = Germinal Epithelium.

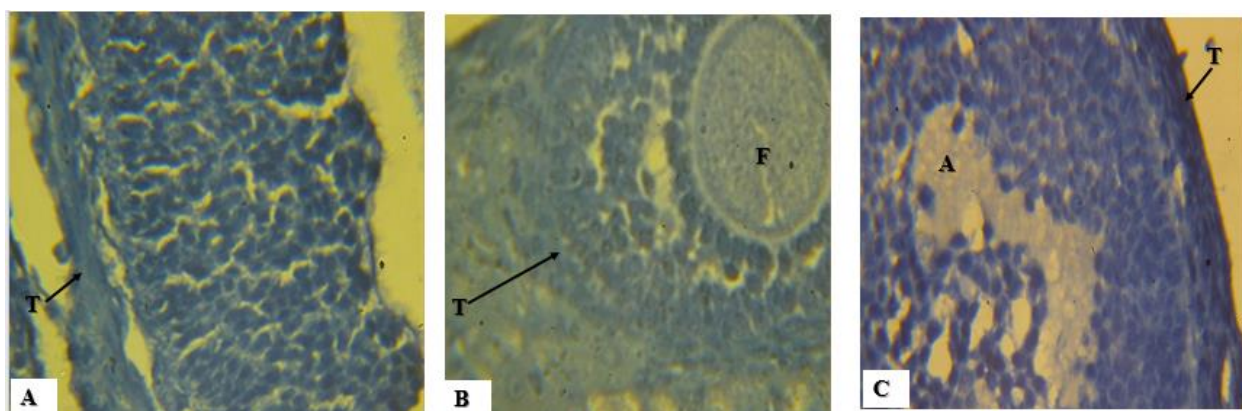


Plate 19: (A) Photomicrograph of ovary from a pregnant rat in the control group stained for Receptor (HER2) [Mag. = x 400]; (B) Photomicrograph of ovary from a pregnant rat in the low-dose nifedipine-treated group stained for Receptor (HER2) [Mag. = x 400] and (C) Photomicrograph of ovary from a pregnant rat in the high-dose nifedipine-treated group stained for Receptor (HER2) [Mag. = x 400].

Keys: T = Theca Cells; F = Follicle; A = Antrum.

4.5.0 HAEMATOLOGICAL OBSERVATIONS

4.5.1 HAEMATOLOGICAL PROFILES OF NON-PREGNANT / PREGNANT RATS IN THE CONTROL, LOW-DOSE NIFEDIPINE-TREATED GROUP AND HIGH-DOSE NIFEDIPINE-TREATED GROUP

Table 9a: Showing the mean values of Pack Cell Volume (PCV) in the haematological profiles of non-pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

PCV (%) [Men = 40 – 52%; Female = 37 – 47%]

Groups	Mean ± SEM
Control	47.83 ± 1.92
Group II	47.67 ± 1.94
Group III	46.17 ± 1.96

Table 9b: Showing the mean values of Pack Cell Volume (PCV) in the haematological profiles of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

PCV (%) [Men = 40 – 52%; Female = 37 – 47%]

Groups	Mean ± SEM
Control	34.00 ± 1.92
Group II	30.00 ± 1.94
Group III	36.80 ± 1.96

Table 10a: Showing the mean values of Red Blood Cell (RBC) in the haematological profiles of non-pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

RBC (x 10¹² / Litre) [Men = 4.5 – 5.9; Women = 4.1 – 5.1; Infants = 4.0 -5.6]

Groups	Mean ± SEM
Control	7.94 ± 0.19
Group II	7.90 ± 0.27
Group III	7.60 ± 0.26

Table 10b: Showing the mean values of Red Blood Cell (RBC) in the haematological profiles of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

RBC (x 10¹² / Litre) [Men = 4.5 – 5.9; Women = 4.1 – 5.1; Infants = 4.0 -5.6]

Groups	Mean ± SEM
Control	5.70 ± 0.19
Group II	5.12 ± 0.27
Group III	6.12 ± 0.26

Table 11a: Showing the mean values of Haemoglobin (Hb.) in the haematological profiles of non-pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

HB (Gram / dL) [	
Groups	Mean $\pm$ SEM
Control	13.69 $\pm$ 0.35
Group II	13.40 $\pm$ 0.35
Group III	14.10 $\pm$ 0.53

Table 11b: Showing the mean values of Haemoglobin (Hb.) in the haematological profiles of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

HB (Gram / dL) [	
Groups	Mean $\pm$ SEM
Control	11.00 $\pm$ 0.35
Group II	9.62 $\pm$ 0.35
Group III	11.30 $\pm$ 0.53

Table 12a: Showing the mean values of Mean Corpuscular Haemoglobin Concentration (MCHC) in the haematological profiles of non-pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

MCHC (Gram /dL) [32 – 36]	
Groups	Mean ± SEM
Control	30.00 ± 0.36
Group II	30.17 ± 0.83
Group III	30.33 ± 0.61

Table 12b: Showing the mean values of Mean Corpuscular Haemoglobin Concentration (MCHC) in the haematological profiles of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

MCHC (Gram /dL) [32 – 36]	
Groups	Mean ± SEM
Control	33.00 ± 0.36
Group II	30.00 ± 0.83
Group III	30.60 ± 0.61

Table 13a: Showing the mean values of Mean Corpuscular Haemoglobin (MCH) in the haematological profiles of non-pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

MCH (pg) [27 – 32]	
Groups	Mean $\pm$ SEM
Control	20.50 $\pm$ 1.65
Group II	21.00 $\pm$ 2.09
Group III	20.17 $\pm$ 1.58

Table 13b: Showing the mean values of Mean Corpuscular Haemoglobin (MCH) in the haematological profiles of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

MCH (pg) [27 – 32]	
Groups	Mean $\pm$ SEM
Control	20.20 $\pm$ 1.65
Group II	18.20 $\pm$ 2.09
Group III	18.20 $\pm$ 1.58

Table 14a: Showing the mean values of Mean Corpuscular Volume (MCV) in the haematological profiles of non-pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

MCV (Fentolitre = fL) [80 – 100]	
Groups	Mean $\pm$ SEM
Control	69.83 $\pm$ 4.22
Group II	69.83 $\pm$ 3.84
Group III	69.17 $\pm$ 2.21

Table 14b: Showing the mean values of Mean Corpuscular Volume (MCV) in the haematological profiles of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

MCV (Fentolitre = fL) [80 – 100]	
Groups	Mean $\pm$ SEM
Control	69.83 $\pm$ 4.22
Group II	69.83 $\pm$ 3.84
Group III	69.17 $\pm$ 2.21

Table 15a: Showing the mean values of White Blood Cell (WBC) in the haematological profiles of non-pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

WBC (x 10⁹ / L) [4,000 – 11,000]	
Groups	Mean ± SEM
Control	10.03 ± 0.32
Group II	8.71 ± 1.59
Group III	7.90 ± 0.64

Table 15b: Showing the mean values of White Blood Cell (WBC) in the haematological profiles of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

WBC (x 10⁹ / L) [4,000 – 11,000]	
Groups	Mean ± SEM
Control	11.70 ± 0.32
Group II	5.62 ± 1.59
Group III	8.76 ± 0.64

Table 16a: Showing the mean percentage of Neutrophil (NEUT.) in the white cell count of non-pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

	NEUT. (%) [40 – 72]
Groups	Mean $\pm$ SEM
Control	21.00 $\pm$ 2.62
Group II	20.00 $\pm$ 0.89
Group III	19.50 $\pm$ 2.70

Table 16b: Showing the mean percentage of Neutrophil (NEUT.) in the white cell count of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

	NEUT. (%) [40 – 72]
Groups	Mean $\pm$ SEM
Control	32.80 $\pm$ 2.62
Group II	33.60 $\pm$ 0.89
Group III	23.20 $\pm$ 2.70

Table 17a: Showing the mean percentage of Lymphocyte (LYMPH.) in the white cell count of non-pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

LYMPH. (%) [20 – 45]	
Groups	Mean $\pm$ SEM
Control	77.00 $\pm$ 3.00
Group II	19.00 $\pm$ 17.00
Group III	76.33 $\pm$ 2.85

Table 17b: Showing the mean percentage of Lymphocyte (LYMPH.) in the white cell count of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

LYMPH. (%) [20 – 45]	
Groups	Mean $\pm$ SEM
Control	67.20 $\pm$ 3.00
Group II	66.80 $\pm$ 17.00
Group III	76.40 $\pm$ 2.85

Table 18a: Showing the mean percentage of Eosinophil (EOS.) in the white cell count of non-pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

EOS. (%) [1 – 6]

Groups	Mean $\pm$ SEM
Control	5.80 $\pm$ 3.56
Group II	1.00
Group III	2.34 $\pm$ 0.95

Table 18b: Showing the mean percentage of Eosinophil (EOS.) in the white cell count of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

EOS. (%) [1 – 6]

Groups	Mean $\pm$ SEM
Control	0.00 $\pm$ 0.00
Group II	0.00
Group III	0.40 $\pm$ 0.00

Table 19a: Showing the mean percentage of Basophil (BASO.) in the white cell count of non-pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

BASO. (%) [0 – 0.5]	
Groups	Mean ± SEM
Control	Nil
Group II	1.00
Group III	1.50 ± 0.50

Table 19b: Showing the mean percentage of Basophil (BASO.) in the white cell count of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

BASO. (%) [0 – 0.5]	
Groups	Mean ± SEM
Control	Nil
Group II	Nil
Group III	Nil

Table 20a: Showing the mean percentage of Monocyte (MONO.) in the white cell count of non-pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

MONO. (%) [2 – 8]	
Groups	Mean ± SEM
Control	1.00
Group II	3.00
Group III	1.33 ± 0.21

Table 20b: Showing the mean percentage of Monocyte (MONO.) in the white cell count of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

MONO. (%) [2 – 8]	
Groups	Mean ± SEM
Control	Nil
Group II	Nil
Group III	Nil

Table 21a: Showing the mean value of Platelet (PLT.) in the haematological profile of non-pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

PLT. ($\times 10^9$/ L) [150 – 400]	
Groups	Mean $\pm$ SEM
Control	2.89 $\pm$ 42.88
Group II	3.4 $\pm$ 40.38
Group III	3.48 $\pm$ 40.91

Table 21b: Showing the mean value of Platelet (PLT.) in the haematological profile of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

PLT. ($\times 10^9$/ L) [150 – 400]	
Groups	Mean $\pm$ SEM
Control	1234.4 $\pm$ 42.88
Group II	883.6 $\pm$ 40.38
Group III	792.8 $\pm$ 40.91

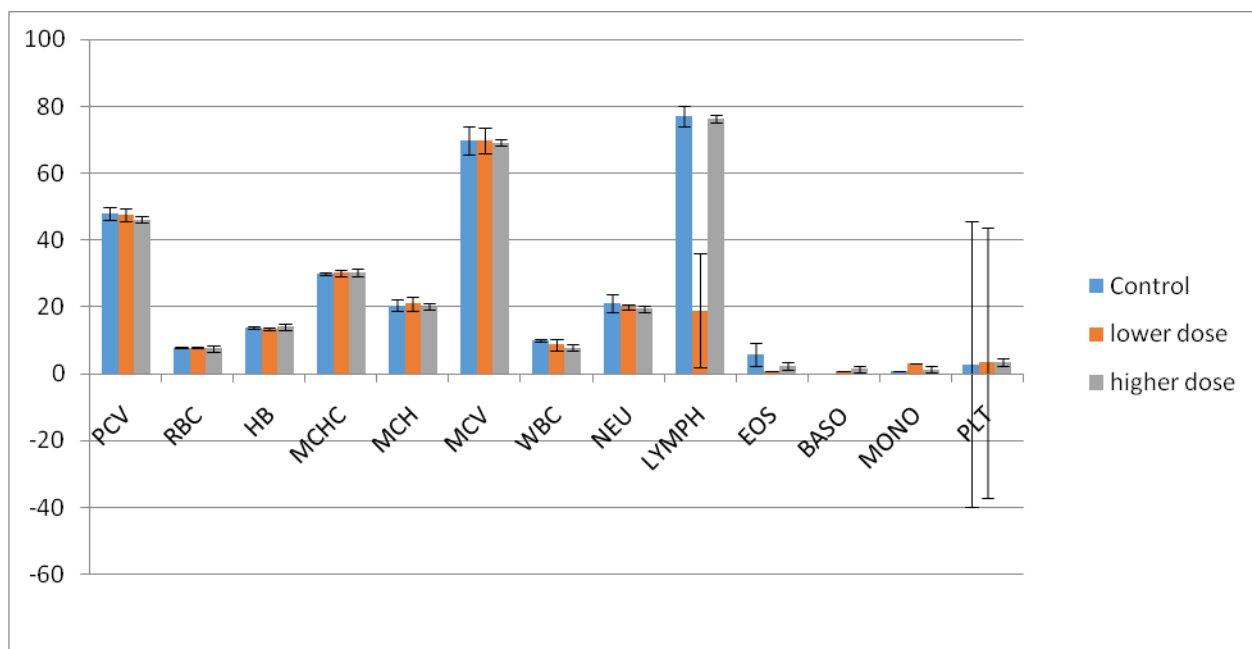


Chart 6: Bar chart showing haematological profile of non-pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

Key: PCV = Packed cell volume; RBC = Red blood cell; WBC = White blood cell; HB = Haemoglobin; PLT = Platelet; MCV = Mean corpuscular volume; MCHC = Mean corpuscular haemoglobin concentration; MCH = Mean corpuscular haemoglobin; NEU. = Neutrophils; LYMPH. = Lymphocytes; EOS. = Eosinophils; BASO. = Basophils; PLT. = Platelets.

4.5.2 HAEMATOLOGICAL PROFILES OF PREGNANT RATS IN THE CONTROL, LOW-DOSE NIFEDIPINE-TREATED GROUP AND HIGH-DOSE NIFEDIPINE-TREATED GROUP

Table 22: Showing the mean values of Pack Cell Volume (PCV) in the haematological profiles of pregnant rats in the control, low-dose nifedipine-treated group and high-dos nifedipine-treated e group.

	PCV (%)
Groups	Mean $\pm$ SEM
Control	34.83 $\pm$ 1.92
Group II	30.67 $\pm$ 1.94
Group III	36.8 $\pm$ 1.96

Table 23: Showing the mean values of Red Blood Cell (RBC) in the haematological profiles of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

	RBC(x 10¹² / Litre)
Groups	Mean ± SEM
Control	5.7 ± 0.19
Group II	5.12± 0.27
Group III	6.12± 0.26

Table 24: Showing the mean values of Haemoglobin (Hb.) in the haematological profiles of n-pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

	HB (Gram / dL)
Groups	Mean $\pm$ SEM
Control	11.0 $\pm$ 0.35
Group II	9.62 $\pm$ 0.35
Group III	11.3 $\pm$ 0.53

Table 25: Showing the mean values of Mean Corpuscular Haemoglobin Concentration (MCHC) in the haematological profiles of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

MCHC (Gram /dL)	
Groups	Mean $\pm$ SEM
Control	33.00 $\pm$ 0.36
Group II	30.17 $\pm$ 0.83
Group III	30.6 $\pm$ 0.61

Table 26: Showing the mean values of Mean Corpuscular Haemoglobin (MCH) in the haematological profiles of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

MCH (pg)

Groups	Mean $\pm$ SEM
Control	20.2 $\pm$ 1.65
Group II	18.2 $\pm$ 2.09
Group III	18.2 $\pm$ 1.58

Table 27: Showing the mean values of Mean Corpuscular Volume (MCV) in the haematological profiles of pregnant rats in the control, low-dose group and high-dose group.

MCV (Fentolitre = fL)

Groups	Mean $\pm$ SEM
Control	59.6 $\pm$ 4.22
Group II	58.4 $\pm$ 3.84
Group III	60.2 $\pm$ 2.21

Table 28: Showing the mean values of White Blood Cell (WBC) in the haematological profiles of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

WBC ($\times 10^9$ / L)	
Groups	Mean $\pm$ SEM
Control	11.7 $\pm$ 0.32
Group II	5.62 $\pm$ 1.59
Group III	8.76 $\pm$ 0.64

Table 29: Showing the mean percentage of Neutrophil (NEUT.) in the white cell count of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

NEUT. (%)

Groups	Mean $\pm$ SEM
Control	32.8 $\pm$ 2.62
Group II	33.6 $\pm$ 0.89
Group III	23.2 $\pm$ 2.70

Table 30: Showing the mean percentage of Lymphocyte (LYMPH.) in the white cell count of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

LYMPH. (%)	
Groups	Mean $\pm$ SEM
Control	67.2 $\pm$ 3.00
Group II	66.4 $\pm$ 17.00
Group III	76.8 $\pm$ 2.85

Table 31: Showing the mean percentage of Eosinophil (EOS.) in the white cell count of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

EOS. (%)	
Groups	Mean $\pm$ SEM
Control	Nil
Group II	Nil
Group III	0.4

Table 32: Showing the mean value of Platelet (PLT.) in the haematological profile of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

PLT. ($\times 10^9$ / L)	
Groups	Mean $\pm$ SEM
Control	1234.4 $\pm$ 2.88
Group II	883.6 $\pm$ 40.38
Group III	792.8 $\pm$ 40.91

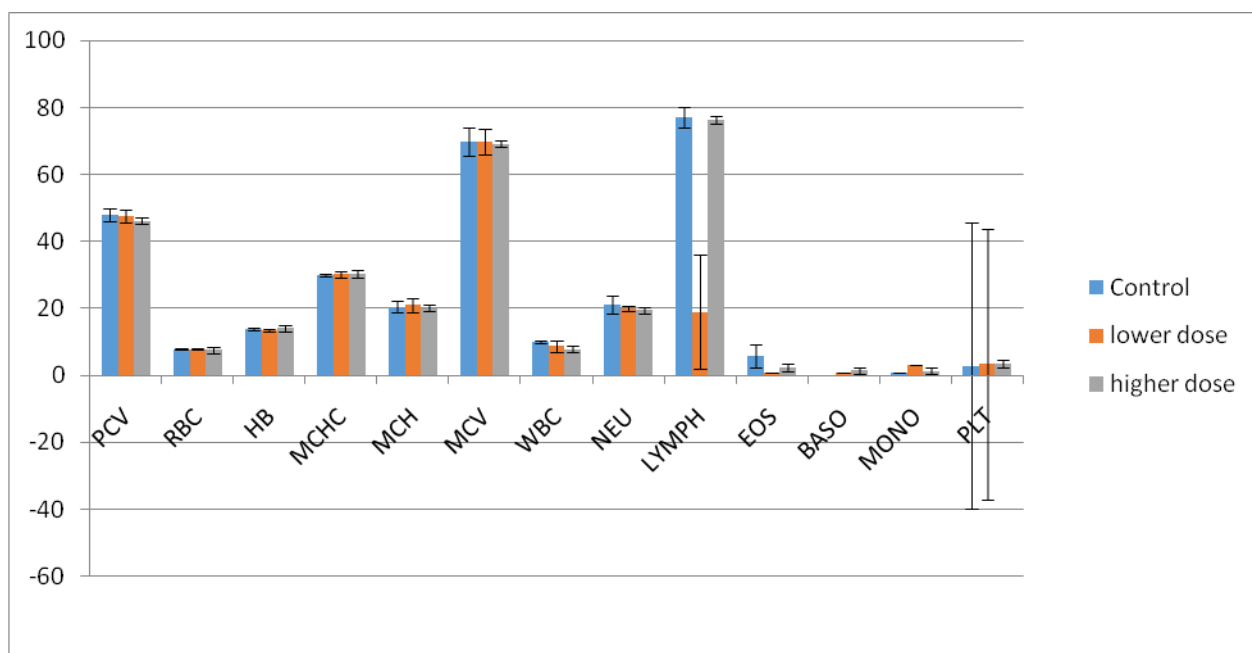


Chart 7: Bar chart showing haematological profile of pregnant rats in the control, low-dose nifedipine-treated group and high-dose nifedipine-treated group.

Key: PCV = Packed cell volume; RBC = Red blood cell; WBC = White blood cell; HB = Haemoglobin; PLT = Platelet; MCV = Mean corpuscular volume; MCHC = Mean corpuscular haemoglobin concentration; MCH = Mean corpuscular haemoglobin; NEU. = Neutrophils; LYMPH. = Lymphocytes; EOS. = Eosinophils; BASO. = Basophils; PLT. = Platelets.

4.6.0 Effects on the foetuses

The uterii were all normally bicornate as expected. They all contained living foetuses during sacrifice. They were fixed intact in 4% PFA and laid open later to examine the contents. Some foetal parameters were assessed and recorded as shown in the **Table 33** below.

Table 33: Showing the foetal parameters in the pregnant rats

	Change	in	pups	body
Treatment groups	bw (g)	No. of pups	weight (g)	
Control	63.43 ± 5.11	8.83 ± 0.70	4.95±0.24	
ADELAT (1.5 mg/kg)	63.15 ± 6.71	10.00 ± 0.37	4.85±0.13	
ADELAT (3.0 mg/kg)	51.46 ± 5.68	11.00 ± 0.55*	4.46±0.15	

Data were presented as Mean ± SEM, * indicates significant increase as compared to the control group at $p < 0.05$. Nifedipine increased the number of pups but not their body weights in pregnant rats.

During and after the administration of the drug, there was no vaginal bleeding. Our study showed that nifedipine had minimal effect on implantation. There were few resorption sites observed in the treated groups. Many fetuses were implanted and viable. There was statistically significant difference in the number of fetuses. No difference observable in the weights of fetuses, umbilical cord lengths and weight of the foetal placentae of the nifedipine-treated and control rats. No gross external abnormality was observed in any of the groups.

4.7 INTERPRETATION

4.7.1 Histological observation

Photomicrographs of the ovary in the non-pregnant control group showed a cross section with normal cortex and medulla which clearly showed varying sizes of follicles, including a corpus luteum.

Photomicrographs of the ovaries in non-pregnant rats of low-dose and high-dose nifedipine-treated group showed increasing reduction in the population of the follicles.

4.7.2 Oestrus cycle observation

The control group had a normal reproductive cycle (4days; Range = 3 to 4days), while the rats in low-dose and high-dose nifedipine-treated groups showed complete disruption in their cycles (Range = 1 – 12 and 1 – 15days respectively; average duration were 5.13 and 4.5days respectively). [Tables A, B & C in Appendix I].

4.7.3 Hormonal values

Progesterone, oestradiol, luteinizing and follicle stimulating hormone were assayed for. The results are displayed in **Charts 2, 3, 4** and **5**. From the results, it shows significant differences in oestradiol, luteinizing hormones, progesterone but no significant difference in follicle stimulating hormone

4.7.4 Haematological observations

The results of the haematological tests carried out are shown in **Tables 9 to 32** and **Charts 6 & 7 (see above)**. The indices showed varying shades of derangements between the nifedipine-treated and the control groups. The RBCs were increased in all groups. This may be so to maintain the ratio 1 to 3 between the haemoglobin concentration and the packed cell

volume as the mean corpuscular haemoglobin and volume were respectively lower than normal! There were neutropenia and lymphocytosis except in the low-dose nifedipine-treated, which recorded slight below-normal lymphocyte status. Basophil percentage of the WBC of the drug-treated groups rose proportionally. Platelet counts were normal in all non-pregnant rats. There were no significant differences in many of the parameters, viz: White Blood Cells (WBC) counts, Platelets (PLT) counts, Mean Corpuscular Haemoglobin (MCH) and Neutrophils (NEUT.).

4.7.5 Effects on the foetuses

The results showed some effects on foetuses: this study showed that nifedipine had minimal effect on implantation as there were few resorption sites observed in the treated groups. Many foetuses were, however, implanted and viable. There was statistically significant difference in the number of foetuses in the nifedipine-treated compared with the control. This increase appeared to be dose-related! No difference was observable in the weights of foetuses, umbilical cord lengths and weight of the foetal placentae of the nifedipine-treated and control rats. No gross external abnormality was observed in any of the groups. There was no vaginal bleeding during and after the administration of the drug.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

The consequences of effects of drugs on any organ of the body could be severe and in most cases, cannot be pre-determined. Any medication could affect the activities of the reproductive system and the hypothalamic – pituitary – gonadal axis will surely have significant effects on the reproductive performances of any individual taking the medication especially the females. The findings in this study showed that there was slight reduction in the weights of the control group compared with the treated groups. The increase in weight might have been due to some reduced activities in which the rats were exposed to and may also be related to some fat accumulation often associated with the use of some of the antihypertensive agents. However, this increase in weight was at variance with the report of Levin and Olivari, which reported that the weight would be significantly less after chronic nifedipine therapy (Levin and Olivari, 1994).

The oestrous cycle in rat is equivalent to the menstrual cycle in human. Oestrous cycle study was important to ascertain if the rats were in cycle to determine their time of ovulation (Long and Evans, 1922). Going by the results obtained for the oestrous cycle in this study, it could be observed that the control group had a normal oestrous cycle (average of 4 days) when compared with those treated with nifedipine. These interferences were irregularity and varying durations of the phases; for instance, the average frequencies of prooestrus, oestrus, metoestrus and dioestrus in this study were respectively 3:4:3:11 and 3:4:4:10 times compared with 6:5:5:5 times in the control. The low-dose and high-dose nifedipine-treated groups had an interference with their oestrous cycles, such that the drug prolonged the duration of the diestrus phase and subsequently lowering the frequency at which the oestrus

phase occurred. Consequently, the frequency of ovulation is reduced and fertility may be, therefore, be impaired. It could therefore be inferred that nifedipine at the doses given in this study could potentially interfere with fertility and could result in menstrual disorders in women of child-bearing age. Nifedipine may interfere with the development of ovarian follicles, resulting in anovular menstrual cycles. Nifedipine can therefore be used as an effective female contraceptive because of possibility of anovular cycles. It can possibly be used to induce an experimental polycystic ovarian syndrome (PCOS) state in laboratory reproductive studies. An actual oval count would have confirmed this observation!

Follicular growth is regulated by endocrine (follicle stimulating hormone, luteinizing hormone and prolactin) and local (paracrine and autocrine) factors. The latter include steroid hormones (such as progestins, oestrogens and androgens) produced by different cell types of the ovary and various non-steroidal regulators (such as oocyte maturation inhibitor, luteinization stimulator, luteinization inhibitor, follicle stimulating hormone inhibitor, insulin-like growth factors, transforming growth factors, epidermal growth factor, platelet-derived growth factor, inhibin and activin) (Hirshfield, 1991; Guraya, 1998; Dhaliwal *et al.*, 1999; Gbotolorun, 2008).

Ovulation, the result of follicular growth, is a complex, multistep process that is triggered, in cycling rats, by the preovulatory luteinizing hormone surge on the evening of prooestrus. This rapid surge of luteinizing hormone begins at about 2–3 p.m. on proestrus and ultimately reaches peak level at 5–7 p.m. on the same evening (Gbotolorun, 2008).

Oestrogen (oestradiol) level was statistically significantly ($p = 0.037$) increased in the low-dose group compared with control group level. Elevated serum oestrogen level stops the follicle stimulating hormone being produced by a negative feedback mechanism, so that only one egg matures in a cycle and stimulate the pituitary gland to release the hormone LH

(Strandring, 2005). Elevated oestrogen concentration could point to weight gain in women as corresponding to the results of the fibroids, heavy or irregular bleeding and infertility. Increasing oestrogen levels inhibit the release of gonadotropin-releasing hormone (GnRH) and of FSH by the adenohypophysis. Inhibin inhibits release of FSH (Strandring, 2005).

Lutenizing hormone was significantly increased ($p = 0.023$) in the low-dose group compared with control group. LH induces ovulation and which results in the development of the corpus luteum. LH concentration of an individual cannot be determined with just a single test. Instead, it has to be done several times over a period of time (Strandring, 2005). It stimulates the ovary to work alongside with insulin to promote testosterone production and high levels of these hormones are likely to produce too much testosterone. Progesterone level is significantly increased in the treated groups compared with the control group. There was no significant difference in follicle stimulating hormone in all the groups. However, the serum levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH) remain low especially in the morning during the oestrus cycle and elevated in the afternoon in the prooestrus phase. Serum oestradiol levels begin to rise at meteoestrus, reaching the highest levels during prooestrus and returning to its baseline during oestrus phase. The secretion of Progesterone will also increase during the meteoestrus and dioestrus but decreases thereafter and the level of the serum progesterone tend to increase at its second peak towards the end of prooestrus phase (Marcondes *et al.*, 2001).

Haematological results showed that nifedipine did not seem to have any major deleterious effects on the blood, based on the blood parameters' values, as their analysis showed no significant differences.

5.2 Conclusions

Although, this study was unable to provide a substantial answer to the microstructure of the ovary, but it had shown that the use of nifedipine could have a deleterious effect on the ovary based ovarian architecture and hormonal analysis. Administration of nifedipine, in the doses used in this study, disrupted the estrous cycles in Wistar rats and has the potential of an ideal antifertility agent. Further studies are needed in both primates and humans, to find out if nifedipine will have similar effects observed in the rodents.

5.3 Recommendations

Due to some limitations, such as difficulty in getting a research work on this topic, and absence of modern requisite equipment, the research work could be considered as a stepping stone towards a greater and better accomplishment. It is however recommended that more research work on this drug and other antihypertensive drugs should be carried out with particular interest on the female reproductive cycle.

1. Further studies are needed to establish the possible dose ranges and do the actual oval count to establish its effect on ovulation.
2. Phase-specific studies are required to establish which phases are affected by the drug.
3. Studies are required to investigate the reversibility of the effects of the drug, after stoppage of its administration.
4. Studies need to be conducted to investigate the effect on the duration of pregnancy
5. A survey of the menstrual profiles of women currently on treatment with nifedipine is recommended to determine how it affects their fertility rate, menstrual cycle, ovulation, health of the foetuses, length of gestation and parturition.

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APPENDICES

APPENDIX I

VAGINAL SMEAR READINGS

Table A: Showing the vaginal smear readings (oestrus cycle) of the non-pregnant control rats.

Day	F1	F6	F8	F9	F11	F18
1.	P	P	p	P	P	P
2.	D	D	D	D	D	D
3.	M	M	M	M	M	M
4.	E	E	E	E	E	E
5.	P	P	p	P	P	P
6.	D	D	D	D	D	D
7.	M	M	M	M	M	M
8.	E	E	E	E	E	E
9.	P	P	p	P	P	P
10.	D	D	D	D	D	D
11.	M	M	M	M	M	M
12.	E	E	E	E	E	E
13.	P	P	p	P	P	P
14.	D	D	D	D	D	D
15.	M	M	M	M	M	M
16.	E	E	E	E	E	E
17.	P	P	p	P	P	P
18.	D	D	D	D	D	D
19.	M	M	M	M	M	M
20.	E	E	E	E	E	E
21.	P	P	p	P	P	P

Key: E- Oestrus, D- Dioestrus, M- Metoestrus, P- Prooestrus [Range 3 – 4days; Average duration = 4days]

Table B: Showing the vaginal smear readings (oestrus cycle) in group II (low dose nifedipine-treated) non-pregnant rats

Days	F2	F3	F10	F12	F14	F17
1.	D	D	E	M	D	M
2.	D	D	D	D	D	D
3.	P	M	D	M	D	D
4.	E	D	P	D	D	P
5.	M	P	E	M	P	E
6.	D	P	D	D	E	D
7.	D	M	D	D	E	D
8.	E	E	D	D	D	M
9.	P	M	E	D	P	P
10.	D	D	E	E	E	D
11.	D	P	D	D	D	D
12.	P	E	D	D	P	D
13.	E	D	D	M	D	E
14.	M	D	E	P	D	D
15.	D	P	M	E	D	D
16.	D	E	D	M	D	P
17.	P	M	M	D	D	D
18.	M	D	M	D	D	M
19.	D	D	D	P	D	D
20.	D	E	D	E	D	P
21.	P	M	P	M	D	E

Key: E- Oestrus, D- Dioestrus, M- Metoestrus, P- Prooestrus [Range 1 – 12days; Average duration = 5.13days]

Table C: Table showing the vaginal smear readings (oestrus cycle) in group III (high dose nifedipine-treated) non-pregnant rats.

Day	F4	F5	F7	F13	F15	F16
1.	M	D	P	D	D	D
2.	E	D	E	M	D	P
3.	M	P	E	D	E	D
4.	D	E	D	D	E	E
5.	D	M	D	M	D	E
6.	D	D	D	E	D	M
7.	D	M	P	E	D	D
8.	D	E	D	D	P	D
9.	E	P	D	D	E	E
10.	M	D	M	D	E	E
11.	E	M	E	D	D	D
12.	E	M	D	D	D	D
13.	E	M	D	M	P	P
14.	M	P	P	D	D	E
15.	D	M	E	P	D	M
16.	D	D	M	M	D	P
17.	P	D	D	M	D	P
18.	E	D	P	M	P	D
19.	M	D	E	P	D	D
20.	M	D	E	D	P	D
21.	D	D	M	D	D	P

Key: E- Oestrus, D- Dioestrus, M- Metoestrus, P- Prooestrus [Range 1 – 15days; Average duration = 4.5days]

APPENDIX II

PREPARATION OF FIXATIVES

Preparation of Paraformaldehyde Fixation Technique

Preparation of 0.4M Phosphate Buffer (PB)

The following procedure was followed:

10.5 gm. of Sodium di-hydrogen orthophosphate dehydrate (BDH, Merck, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) was added to 500 ml of distilled water. The mixture was stirred to dissolve the solution, followed by addition of 56gm di-potassium hydrogen ortho phosphateanhydrous (BDH, Merck).

This was stirred to dissolve the mixture and then 500 ml of distilled water was added.

Preparation of 4% Paraformaldehyde in 0.1M Phosphate Buffer.

For a litre:

750 ml of distilled water was heated to 60°C

APPENDIX III

TISSUE PROCESSING FOR PARAFORMALDEHYDE-FIXED TISSUES PREPARATION OF TISSUE FOR MICROSCOPIC ANALYSIS

Fixation: After the organ had been harvested from the sacrifice, it was placed in 4% paraformaldehyde which served as a fixative, to prevent tissue damage due to autolysis and putrefaction.

Dehydration: The fixed tissue was then dehydrated in ascending grades of alcohol from 70% (3 hours) then 90% (3 hours). It was then placed in two changes of 100% alcohol, (absolute 1 and 11) at intervals of 2hours each. This was done to remove water from the tissue for effective wax impregnation.

Clearing: The dehydrated tissue was placed in xylene, a clearing agent, for a period of overnight i.e. 14hours to ensure complete clearing. Because xylene is miscible in both alcohol and wax; it was used to remove the dehydrating fluid (alcohol) from the tissue in preparation for filtration.

Infiltration: The cleared tissue was then placed in two changes of molten paraffin wax at 60°C, in the oven, for 1 hour per change.

Embedding: Molten wax was poured into plastic embedding mould, into which the tissue was placed for embedding. The tissue was then transferred into the embedding mould; aligned in such a way that the required surface faced upward. The wax was then allowed to solidify. In order to avoid mixing up of tissues, the mould was labelled using a paper strip, with the area labelled facing downwards.

Trimming: The shiny base of each tissue block was slightly trimmed to reduce excessive wax using a warm knife.

Mounting: This was done simultaneously using heated spatula to press them together. The shiny surface of the tissue blocks were allowed to face upwards while the wooden blocks attached to the opposite surface. Meanwhile, the paper tag meant for identification was still attached in between. Justification for wooden blocks was to serve as a supporting medium for holding the tissue blocks in place for easy sectioning.

Sectioning: The mounted tissue was then put in ice for few minutes to allow it to swell up so as to produce ribbons during sectioning. Meanwhile, the microtome knife was sharpened with an auto-sharp and fixed in position at 45°. Finally, the tissue blocks were sectioned until fine ribbons were collected and placed on plain white sheets of paper with the aid of forceps.

Floating out sections: The shiny surfaces of the sections were laid on trough of cold water in order to allow the sections to float out. Floated sections were then transferred from the cold water into water – bath, maintained at a temperature well below the melting point of paraffin wax. This was done in order for sections to spread better and prevent folding.

Mounting sections on slides: Floated sections were mounted on slides following these steps:

- i. Egg albumen was smeared on cleaned glass slides.
- ii. Each albumenized slide was then used to pick well spread sections from the heated water - bath.
- iii. Subsequently, the slides were placed on a hot plate with a temperature of about 40°C to enhance quicker adhesion of sections on the slides.
- iv. Finally, each slide was labelled and kept on staining racks for staining.

Staining: The stains used were haematoxylin and eosin stains (H and E) (Parry, 2012). Once a tissue specimen has been processed by a histology laboratory, and transferred onto a glass slide, it needs to be appropriately stained for microscopic evaluation. This is because unstained tissue lacks contrast: all of the fixed materials have a similar refractive index and a similar colour. If you viewed an unstained tissue section under the microscope, everything would appear a uniform dull grey colour.

The staining process therefore makes use of various dyes that stain particular cell components within tissues, so that you can distinguish different cell parts from each other.

Haematoxylin and Eosin

For routine examination, haematoxylin and eosin (H&E) is the stain of choice. This stain produces colours for different tissue structures, which would otherwise be transparent, so that you can get a detailed view of the tissue.

As its name suggests, H & E stain makes use of a combination of two dyes – haematoxylin and eosin. The two stains were independently introduced in 1865 and 1875, respectively, by Böhmer and Fischer. In 1876, Wissowzky described their use in combination as a tissue staining method for staining different materials different colours.

Despite its simplicity, this stain has stood the test of time. Even now, over a century later, H & E remains the most frequently used tissue stain worldwide.

The Science Behind H & E

Staining does not produce colour randomly; instead, the dyes exploit differences in the chemistry of the tissue to differentially colour various components.

Ionic bonding is the most important type of bonding that occurs in histological staining techniques. It involves electrostatic attraction between ions of opposite charge, one of which is fixed in the tissue, and the second of which is in the dye.

Haematoxylin alone is not technically a dye, and will not directly stain tissues. It therefore needs to be used in combination with a “mordant” – a compound that helps it link to the tissue. The mordant used is typically a metal cation, such as aluminium. Haematoxylin in complex with aluminium salts is cationic and acts as a basic dye. It is positively charged and

can react with negatively charged, basophilic cell components, such as nucleic acids in the nucleus. These stain blue as a result.

Eosin is anionic and acts as an acidic dye. It is negatively charged and can react with positively charged, acidophilic components in the tissue, such as amino groups in proteins in the cytoplasm. These stain pink as a result.

H & E is a useful all-purpose stain that is also quick and easy to use, which may explain why it has stood the test of time.

Staining procedure: The racks were passed through the following solutions as follows:

- i. Slides were de-waxed in xylene for 3-5 minutes
- ii. The slides were transferred to absolute alcohol for 3 minutes to remove the clearing agent.
- iii. Hydration was further achieved by passing the slides through descending grades of alcohol 90%, 70% and 50% at one minute interval.
- iv. After this, the slides were washed thoroughly in water bath. These above procedures are otherwise known as bringing sections to water.
- v. The tissues were then stained in haematoxylin for about 12-15 minutes. This stained the nuclei black.
- vi. The slides were rinsed under the tap water for 5 minutes in order to reduce haematoxylin concentration and obtain a blue-black natural colour of haematoxylin stain.
- vii. The prepared 1% alcohol was then used to differentiate the tissues, removing excess haematoxylin stains and this was done by dipping the stained slides in 1% acid

alcohol for two seconds. This resulted in fading colours of the tissues and this is also known as regressive staining.

- viii. The tissues were then taking back to water for about 15 minutes and this is known as blueing. This returned the tissues back to their original coloration.
- ix. The tissues slides were then rinsed in water - bath.
- x. Tissues were then dehydrated through ascending grades of alcohol 50%, 70%, 90% absolute I and II, in quick succession of 15 minutes, to prevent the removal of the stains.
- xi. Finally, tissues slides were cleared off by alcohol, by passing them through xylene to ensure transparency of sections and make the tissues clear under microscope examination.

Covering the slides with cover slips: This is done by dipping the stained slides; a drop of DPX (dibutyl phenol in xylene) was mount on the smeared on each slide to serve as a gum for the cover slips. The slides were covered by putting the cover slips on white piece of paper and then attaching the surface of each tissue slides to the cover slips, thereby pressing the slides against the cover slips at the surface. The slides were then viewed under the microscope.

APPENDIX IV

TISSUE STAINING TECHNIQUES:

PREPARATION OF STAINS

(i) 70% ROH = 70 ml ROH+30 ml of distilled water

(ii) 90% ROH = 90 ml ROH+10 ml of distilled water

(ROH=alcohol)

(iii) Eosin Yellow solution (2% aqueous Eosin solution) = 2 g of eosin yellow in 100 ml of distilled water

(iv) Haematoxylin solution

Absolute ROH was added to 8 g of solid haematoxylin, until it was up to 400 ml. Then 400 ml of glycerol was added and warmed in a water bath and shaken continuously. 12 g of KASIO_4 was dissolved in 100 ml of water and added to the earlier prepared solution and well shaken. Then 25ml of acetic acid was added and shaken well. The solution was then exposed under sunlight for several days for ripening. The prepared haematoxylin and eosin solutions were filtered separately with filter paper.

(v) Leishmann: 0.15 g powder, 100 ml methyl alcohol (Methanol)

Leishmann solution: 400 ml of methanol was added to 1g of Leishmann powder in a container.

(vi) Eosin solution: 750 ml of distilled water was added to 7.5 g of eosin powder in a container.

APPENDIX V

IMMUNOHISTOCHEMICAL TECHNIQUES

Immunohistochemistry (IHC) involves the process of selectively imaging [antigens](#) (proteins) in cells of a tissue section by exploiting the principle of [antibodies](#) binding specifically to antigens in [biological tissues](#) (Ramos-Vara & Miller, 2014). IHC takes its name from the roots "immuno", in reference to antibodies used in the procedure, and "histo," meaning tissue (compare to [immunocytochemistry](#)). [Albert Coons](#) conceptualized and first implemented the procedure in 1941 (Coons, Creech & Jones, 1941).

Immunohistochemical staining is widely used in the diagnosis of abnormal cells such as those found in [cancerous](#) tumors. Specific molecular markers are characteristic of particular cellular events such as proliferation or cell death ([apoptosis](#)) (Whiteside & Munglani, 1998). Immunohistochemistry is also widely used in basic research to understand the distribution and localization of [biomarkers](#) and differentially expressed proteins in different parts of a biological tissue.

Visualising an antibody-antigen interaction can be accomplished in a number of ways. In the most common instance, an antibody is conjugated to an enzyme, such as [peroxidase](#), that can catalyse a colour-producing reaction (Ramos-Vara, 2005). Alternatively, the antibody can also be tagged to a [fluorophore](#), such as [fluorescein](#) or [rhodamine](#).

APPENDIX VI

HORMONAL ASSAYS

Immunoenzymometric assay: the essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, in excess, and in native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-hormone assay for (LH, progesterone, FSH) antibody, upon mixing monoclonal biotinylated antibody, the enzyme label antibody and a serum containing the native antigen reaction results between the native antigen and antibodies without competition or steric hindrance to form a soluble sandwich complex. Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody.

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

TEST PROCEDURE

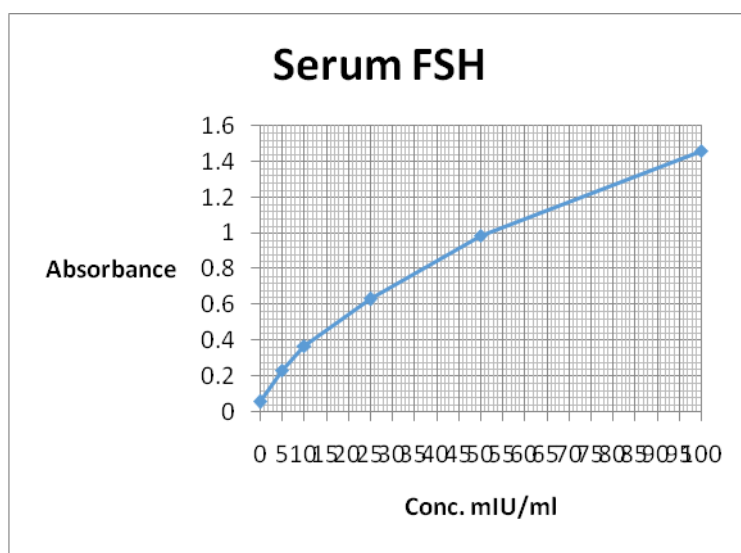
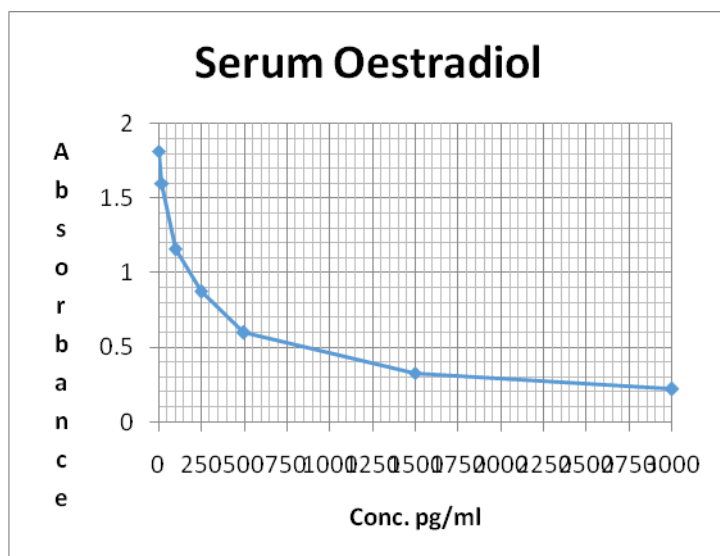
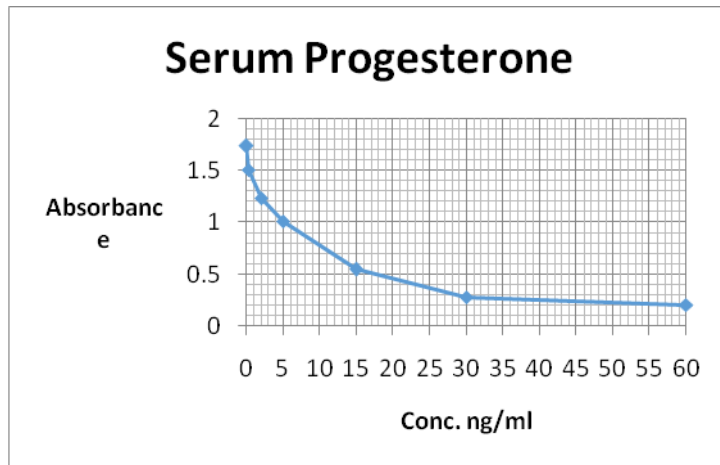
1. Format the microplate wells for each serum reference, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips into the aluminium bag, seal and stored at 2-8°C.
2. Pipette 0.050ml (50ul) of the appropriate serum reference, control or specimen into the assigned well.

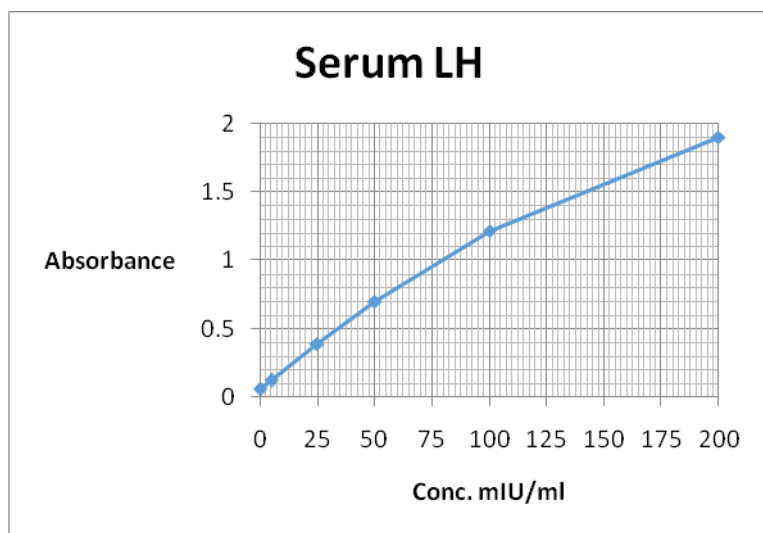
3. Add 0.100 ml (100 ul) of the assay hormone-Enzyme Reagent to all wells.
4. Swirl the microplate gently for 20-30 seconds to mix and cover.
5. Incubate 60 minutes at room temperature.
6. Discard the contents of the microplate by decantation or aspiration. If decanting blot the plate dry with absorbent paper.
7. Add 350 ul of wash buffer decant or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used.
8. Add 0.100 ml (100ul) of working substrate solution to all wells, always add reagents in the same order to minimize reaction time differences between wells.
9. Incubate room temperature for fifteen (15) times.
10. Add 0.050 ml (50ul) of stop solution to each well and gently mix for 15-20 seconds.
11. Read the absorbance in each well at 450 nm (using a reference wavelength of 620-630 nm to minimize well imperfections) in a microplate reader. And the result should be read within 30 minutes of adding the stop solution.

CALCULATION OF RESULTS

1. The mean absorbance value (A_{450}) for each set of reference standards, controls and samples are calculated.
2. A standard curve is plotted by plotting the mean absorbance obtained for each reference standard against its concentration in ng/ml on a linear-linear graph paper, with absorbance values on the vertical or Y axis, and concentrations on the horizontal or X axis.
3. The mean absorbance for each specimen is used to determine the corresponding concentration of the hormones in ng/ml from the standard curve.

Graphs showing the standard curve used for the calculation of the serum progesterone, oestradiol, luteinizing hormone and follicle stimulating hormone.





APPENDIX VII

Fixation Methods

The brain will begin to deteriorate as soon as its blood supply is interrupted. The deterioration is rapid, and it is, therefore, important to arrest the deterioration as quickly as possible. The process of preserving the brain is called "**fixation**".

The two most common ways of fixing the brain are freezing and the use of fixative solutions. Each method has its advantages

FREEZING

Freezing is a much quicker way of fixing the brain and tends to preserve more of the brains biochemistry, but the tissue is trickier to deal with as it must remain frozen until it is on the microscope slide. There is much blood left in the tissue. Freezing is not appropriate if you intend to do immunocytochemistry. It is good for glycogen phosphorylase staining, AChE staining, Mao staining, and Nissl staining (cresyl violet or thionin). Frozen sections can be mounted on plain glass microscope slides.

The rat brain can be frozen by placing it in the freezer or in the chamber of the cryostat-microtome, but for best results rapid freezing is required. Two common methods of rapidly freezing the brain are dry ice and liquid nitrogen. In our lab we use **methyl butane** (isopentane) cooled to -72 degrees C. in a ultra cold freezer. A covered jar of methyl butane is placed in the freezer 20-30 min prior to sacrificing the rat. The rat is then anaesthetized and decapitated. The brain is quickly removed from the skull and immediately immersed in the cold methyl butane. The brain is frozen within a few seconds. It is then wrapped in aluminium foil and stored in the freezer until sectioning.

FIXATIVE SOLUTIONS

The most commonly used fixatives are formalin, glutaraldehyde, and alcohol. A 10% formalin solution is adequate for most purposes, but many stains require specaillized perfusions where pH and osmolarity are carefully controlled.

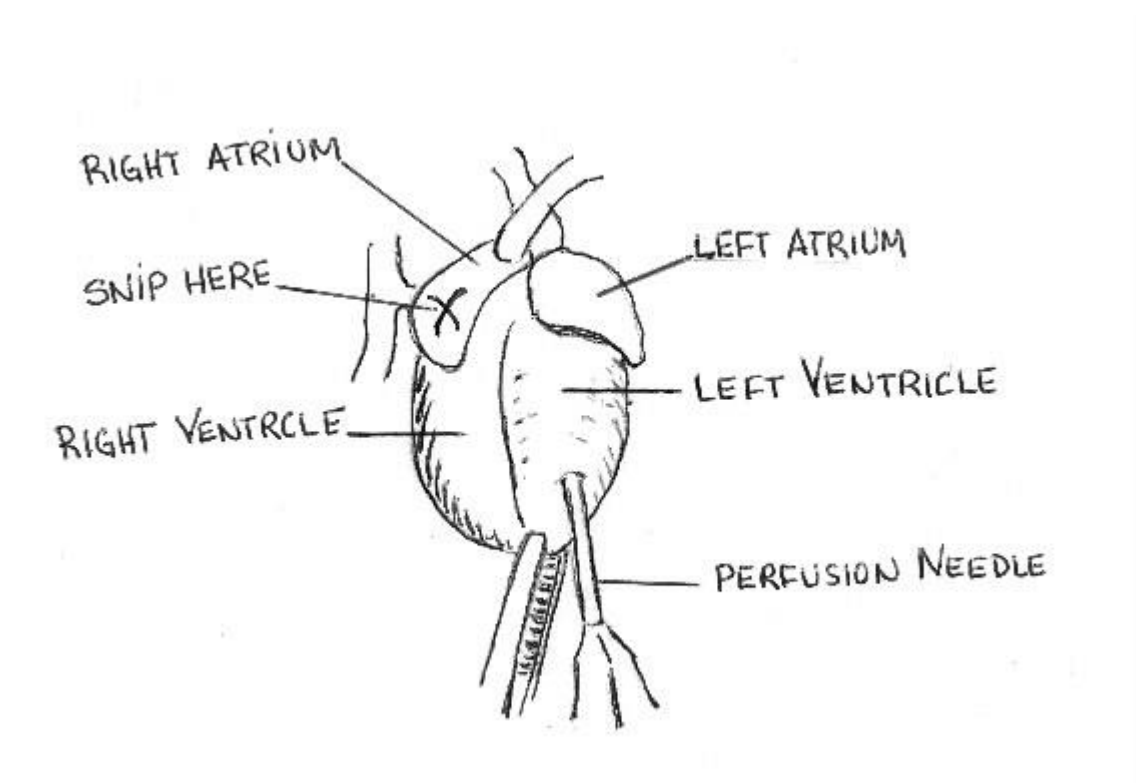
Fixing the brain with fixative solutions takes longer, but the brain is much more resilient and easier to handle. When perfused, most of the blood is removed from the tissue. Fixative Solution are used for immunocytochemistry, HRP staining, and biocytin staining. Nissl stains can also be used on tissue fixed in fixative solutions. Fixative solutions are also used if the tissue is going to be embedded in paraffin or plastic and for vibratome sectioning. Tissue fixed with fixative solutions should be mounted on gelatin coated microscope slides if the sections will be processed after the tissue is on the slides.

Tissue can be fixed in two ways: by **immersion** and by **perfusion**. Immersion is suitable only for small or thin pieces of tissue. In the immersion method the tissue is simply soaked in the fixative solution. The immersion method is often used to fix frozen sections that have been mounted on slide. The slides are dipped in formalin or alcohol for a few minutes to fix them after sectioning and staining. Immersion is not a suitable fixation for larger pieces of tissue such as a whole rat brain. The outside of the brain fixes and impedes the penetration of the

fixative to the centre of the brain. For whole rat brain, the perfusion method is used. In the perfusion method, the circulatory system is cleared of blood and used to circulate the perfusants uniformly through the brain and body.

Perfusion Method

1. anaesthetize the rat.
2. Lay the rat on dorsal surface on a rack above a sink.
3. Make a lateral incision of about 6 cm just below the sternum.
4. Attach a pair of haemostatic forceps to the Xiphoid process.
5. Pull up on the forceps to view the diaphragm.
6. Using scissors poke a hole in the diaphragm close to the Xiphoid process. Then cut the diaphragm away from the ribs. You should be able to see into the thoracic cavity.
7. Using heavy shears, make two longitudinal cuts, one on each side of and about 1 cm lateral to the haemostatic forceps. Be careful to avoid damaging the heart or lungs. The chest can be exposed by pulling the haemostatic forceps up over the head.
8. The heart is covered with a membrane, the pericardium. Use forceps to tear away the pericardium.
9. With blunt forceps, grab the heart at the apex.



10. Using the needle make a hole in the right atrium to drain fluids.
11. Immediately insert the perfusion needle into the left ventricle.
12. Perfuse with about 200 ml normal saline to clear the blood from the circulatory system. The eyes of albino rats will turn clear.
13. Perfuse with about 500 ml of 10% formalin or fixative of choice.
14. Decapitate, remove brain from cranium and store in fixative until sectioning.

Note: The perfusion needle is connected to a pump, which pumps the perfusants through the body. The operation of the pump is not covered here. The operation of the pump must be demonstrated.