THE EFFECTS OF EMBELIN (a Benzoquinone compound of plant origin) ON SOME REPRODUCTIVE PARAMETERS OF FEMALE SPRAGUE-DAWLEY RATS.

BY

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DECLARATION

I declare that the work presented in this thesis is my original work and has not been submitted in this or any other form for a degree in any other university.

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DEDICATION

This work is dedicated to my wife (Ivournie Muwowo) and my daughter (Ndanji Namukoko) for thinking about me all the time while I was away.

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ABSTRACT

Embelin is a plant extract widely used as a herbal medicine against a wide range of ailments in humans. Its use has been associated with sexual debility in male subjects. Studies have shown that it actually posses anti-fertility properties in male laboratory animals. In female rats, it has been shown to cause fetal resorption and to reduce ovarian weights. However, studies in the female have not explicitly elucidated the changes that take place at endocrinological level; neither have they described ovarian morphological changes nor the evolution of estrous cycles associated with embelin exposure. This study aimed to accomplish the above.

The effects of embelin on the evolution of estrous cycles, progesterone and estradiol profiles and ovarian histopathology were examined. Thirty adult (4 months old) regularly cycling female Sprague-Dawley rats were used. They were given 1 ml/kg bwt. Corn oil (groupI-control, n=10); or embelin dissolved in corn oil at 10 mg/kg bwt. (groupII-experimental, n=10) and 20 mg/kg bwt. (groupIII-experimental, n=10). The treatments were given subcuteneously on alternate days for 20 days. Thus, each animal received a total of 10 injections by the end of the experiment. Oestrous cycles were monitored daily by vaginal cytology. Ovarian histopathology and progesterone and estradiol levels were assessed by standard procedures.

Subcutaneous injections of embelin in this animal model resulted in the disruption of the regular oestrous cycle patterns. Specifically, 3 animals out of 10 in group II (10 mg/kg bwt.) and 4 animals out of 10 in group III (20 mg/kg bwt.) essentially remained in dioestrus by the fifth injection. There was a significant depression of plasma estradiol ($P<0.05$) and progesterone ($P<0.02$) at both dose levels. Histopathological evaluation of the ovaries from the embelin treated rats revealed the presence of abnormal follicles. In most follicles, the oocytes showed distortions in their symmetries. There were no significant changes in the liver and pituitary glands.

In a separate experiment, mixed ovarian cells were isolated from another set of normally cycling rats and directly challenged with embelin in vitro.

Results from theses studies revealed that embelin also suppresses estradiol and progesterone secretion by isolated mixed ovarian cells.

These data demonstrate that embelin suppresses plasma estradiol and progesterone levels; disrupts the evolution of the regular oestrous cycles and also affects ovarian morphology in rats. The results further demonstrate that embelin probably exerts its effects by acting directly on ovarian cells.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction.

Experience from users of embelin (*Embelia ribes*), a plant extract used as a herbal remedy for many ailments in humans, has shown that it causes sexual debility in male subjects. This, perhaps, is what prompted reproductive scientists to begin evaluating its effects on reproductive parameters in male laboratory animals in a bid to verify or disprove these claims. Emphasis has mainly been placed on the male reproductive system where results in laboratory animals have revealed that embelin actually possesses anti-fertility properties by reducing gametogenic counts (Gupta *et al.*, 1989; Githui *et al.*, 1991; Mungai *et al.*, 1997). This empirical evidence has led to the notion that embelin could be considered as a potential candidate for a future male contraceptive. In an era where negative socio-cultural tendencies (Africa in particular) hamper population control strategies (e.g. the pill), the idea of a potential male contraceptive is likely to be attractive to birth control practitioners. The assumption is that population control would only be meaningful if active male participation in family planning was guaranteed.

It is probably for this reason that research on embelin (with regard to the

reproductive system) has mainly been directed toward the male; with what can only be described as scanty information on the female reproductive system. But what effects does embelin have on female reproductive parameters? Studies which have been conducted so far (Bargava and Dixit, 1985; Prakash and Vashney, 1980) have shown that embelin causes fetal resorption and loss of ovarian weights in female rats. However, these studies have not considered the very important aspect of how the sex steroid hormone profiles are affected in female animals after exposure to embelin. Knowing the sex steroid hormone profiles in the female is very vital because it gives an indication as to the general integrity of the hypothalamus-pituitary-gonadal axis and may serve to explain some of the observations made so far on the effects of embelin in female laboratory animals.

1.2. Objectives.

The objectives of this study were:

1. To investigate the effects of embelin on female sex steroid hormone profiles in Sprague-Dawley rats.
2. To investigate the effects of embelin on the oestrous cycles of these rats.
3. To investigate the effects of embelin on ovarian morphology, and;
4. To determine the effects of embelin on isolated rat mixed ovarian cells.

1.3.0. Literature Review

1.3.1. Background information

Nature and distribution of embelin

Embelin is a naturally occurring compound which is obtained from plants of the family Myrcinaceae. It belongs to the benzoquinone group of chemical compounds. When extracted from its plant sources, it appears as a reddish-brown crystalline compound. (Ogawa and Natoris, 1968; Midiwo *et al.,* 1988). These plants are distributed in many parts of the world, particularly hilly regions including parts of Kenya and Tanzania. Embelin occurs in the berries, barks and leaves of *Ardisia humilis* (Brabhu, 1971), stems of *Embelia tsjersimu-cottam,* fruits of *Rapanea umbellata* and also in the roots of *Connanus ritchkei.* Other species in the family myrcinaceae include *Embelia ribes, embelia robusta* etc. These are all climbing plants.

Uses of embelin.

Embelin is an example of a herbal medicine. According to Webster`s Third New International Dictionary a herb is defined as a plant or plant part valued for its medicinal, savory, or aromatic qualities. Herb plants produce and contain a variety of chemical substances which act upon the body in different ways. Perhaps herbal medicine is the oldest form of health care known to man. Herbs have been used by all cultures throughout history.

Many of the drugs used today are of herbal origin (e.g. quinine from the bark of cinchona tree) and the majority of the prescription drugs dispensed today in

many parts of the world contain at least one active ingredient derived from plants. Some drugs are made from plant extracts; others are synthesized to mimic a natural plant compound. The World Health Organization (WHO), 1994 estimates that about 4 billion people, approximately 80% of the world population, presently use herbal medicine for some aspect of primary health care.

Embelin is used as a remedy for many ailments by local people in places where it occurs. It is known by different names in different parts of the world. For example, in India, Himalayas, Srilanka and Singapore, it is called Viranga, while in East Africa it is called Gezi, Igalilonde or Ngera (Kokwaro, 1993). It is used as an alterative, (medicine that alters the process of nutrition) antihelmintic, stimulant etc. Leaves are used to treat swollen breasts of mothers, or by women having difficulties with birth. The fruits are used as both purgative and vermifuge. Both dried fruits and roots are boiled or soaked in water and the infusion drank for intestinal worms Kokwaro (1993).

Physical and chemical properties.

Embelin has the following chemical formular: C17H26O4; molecular weight: 294 kd. It is a lipid soluble compound. It is usually extracted from plants in its pure form (100%) but sometimes it may occur with other benzoquinone compounds such as rapanone, which makes it difficulty to resolve (Thompson, 1987).

Benzoquinone compounds can be found abundantly in nature. They occur in plants, arthropods, and fungi. Specifically, those found in arthropods include p-benzoquinone and very simple alkyl derivatives. The alkyl derivatives do not occur anywhere else except in reduced form. P-toliquinone and the hydroquinone occur in the ascomycetes, *Necteria erubescens*. (Thompson, 1987).

The animal of study (Sprague-Dawley rat)

Biology of reproduction of the female rats.

The ovary

The reproductive system in the rat consists of the paired ovaries, the paired uterine horns, the uterus, the cervix, vagina, vestibule and external genitalia. The ovaries are located in the posterior dorsal part of the abdominal cavity. They are small, irregular, retroperitoneal structures suspended from the dorsal abdominal wall by a ligament, the mesovarium, and are held in place by the pressure of surrounding organs. They produce the female germ cells (ova) and female sex hormones (estrogens and progesterone). They are embryologically homologous with the testis of the male.

The ovary is composed of a loosely organized central portion or medulla, and a denser outer portion or cortex which is covered by a connective tissue envelope (tunica albuginea) and a thin mesothelium (germinal epithelium). The medulla is covered by the cortex, except at the hilus where the blood vessels, nerves and lymphatics enter and leave the gland. The cortex and medulla are not separated

by a distinct line of demarcation but blend together at the indistinct cortico-medullary border. The cortex is the region where growth of ova and development of follicles and corpora lutea takes place. Each ovum as it ripens is surrounded by a gradually enlarging fluid-filled sac, or follicle. Several mature follicles (Graafian follicles) rapture at estrus and expel mature ova into the dilated (pavilion) end of the oviduct. The raptured follicles then pass through three stages, the corpus heamorrhagicum, the corpus luteum (either of estrus or pregnancy), and the corpus albicans, which is a non-functional scar tissue (Harrison,1962; Bloom and faucett, 1966)

The follicular complex

Reproductive aberrations by exogenous compounds in the female reproductive system may not only be effected at physiological or molecular level but also at morphological level, particularly on important elements within the reproductive system.

The follicular complexes are structurally the most conspicuos and functionally the most important elements in the ovary. The microscopic appearance and arrangement of components in follicular complexes undergo changes coincident with growth and development of these structures during fetal and post natal life. These changes are sufficiently characteristic that adjectives such as "primordial", "primary" and "graafian" convey meaning about morphology of components during follicular growth and development in normal and pathophysiological states. Components of the follicular complexes include the

oocytes, granulosa cells and theca cells (Franchi *et al.*, 1962).

The oocyte

The oocyte is the fundamental element of both the structural and functional integrity of the follicle complex. When the oocyte degenerates, other follicular components either die or "dedifferentiate" and steroid hormone synthesis and secretion are changed, both quantitatively and qualitatively.

The progenitor of the oocyte is an oogonium which arises from primordial germ cell. Primordial germ cells are known to migrate actively and freely from the yolk sac to the genital ridge where they undergo successive mitotic divisions to give rise to oogonia. Soon, most of the cell divisions cease and initiation of the first meiotic division marks the conversion of these cells into primary oocytes. After oocyte growth is completed, follicular growth continues. At ovulation, the oocyte and surrounding cells in the cummulus oophurus are extruded. The completion of the first meiotic metaphase and formation of the first polar body convert the primary oocyte into the secondary oocyte. After another nuclear division, the second polar body is eliminated. As a result of two nuclear divisions, with only one replication of the chromosome, the ovum nucleus contains a haploid number of chromosomes at the time of sperm penetration at fertilisation (Franchi *et al.*, 1962)

Granulosa cells

The primary oocytes become closely associated with a single layer of spindle-

shaped cells, which are the precursors of granulosa cells. They may originate from the rete ovarii. Cytoplasmic processes from these cells form unions with the plasma membranes of the oocyte ultrastructurally similar to desmosomes. The oocyte and the associated granulosa cells are separated from the surrounding stroma by a membrane, the "basal lamina". This complex, of an oocyte and spindle-shaped cells enclosed by the basal lamina, is called a "primordial follicle". Follicular maturation is initiated when the spindle-shaped granulosa cell precursors in some primordial follicles differentiate into a single layer of cuboidal cells that then begin to divide.

In such follicles, called "primary follicles", granulosa cell proliferation gives rise to multiple layers of cells which contribute to enlargement of the follicle. Granulosa cells (and the oocyte) synthesize and secrete glycoproteins, which give rise to a translucent halo called the "zona pellucida" that surrounds the oocyte. The cytoplasmic processes of the granulosa cells traverse the zona pellucida to maintain intimate contact with the plasma membrane of the oocyte.(Bivin *et al.*, 1979).

Theca cells

Shortly after granulosa cells begin to proliferate in primary follicles, changes can be observed in the cortical stromal cells outside the basal lamina. These changes consist of a concentric layering and a decrease in nuclear density of spindle-shaped cells called "theca cells", and proliferation of lymphatics and blood vessels which terminate at the basal lamina. As follicular maturation

progresses, increasing proportions of these spindle-shaped theca cells proximate to the basal lamina , accumulate cytoplasm, become epethelioid in appearance, acquire organelles characteristic of steroid hormone-secreting cells, and are referred to as "theca interna" cells. More peripheral layers of these cells retain their spindle-shaped configuration and merge with stromal cells; these are called "theca externa" cells (Bivin *et al.*, 1979)..

Reproductive physiology

Estrus

In normal mature female mammals, the dynamic events taking place at physiological and molecular level within the reproductive system influence the expression of some discernable manifestations of such events. In lower mammals these processes are manifested outwardly by estrous signs or heat, while in primates they are manifested by menstrual flows. Aberrant estrous cycles and menstrual cycles will almost always be extrapolated to a defect within the reproductive system at physiological and/or molecular level. When evaluating the consequences of a potentially debilitating substance on the female reproductive system, reproductive biologists make use of the alterations exhibited phenotypically in the estrous or menstrual cycles as one way of establishing whether or not the substance is a reproductive toxin. In this regard, appreciating the normal cycle of the animal of study is imperative.

Sexual maturity in rats normally occurs between six and seven weeks from birth

for both sexes, although the onset of first estrus in females occurs at about five weeks from birth. The vagina opens between 34 and 109 days of age, although the vaginas remain fully retractable in adults. Ovulation in rats can be induced by forced estrus during non-estrus intervals. Vaginal stimulation is important in rat reproductive physiology since ovulation in the rat depends on this.

The rat is a polyoestrous animal. It repeats its cycles throughout the year without much variations unless interrupted by pregnancy. Induced ovulation in the rat dependent on a series of neuroendocrine events (Goldenberg *et al.*, 1972). The estrous cycle in the rat is 4-5 days in length (Louvet and Vaitukaitis, 1976), divisible into four stages as exemplified by vaginal cytology. The structural aspects of the oestrous cycle of the rat have been very carefully analyzed. Robert and Moore (1978), summarised the various stages as follows:

Oestrus-this is the period of heat during which the levels of estrogens are high. Females in oestrus are hyperactive and brace themselves when touched. Their ears quiver when they are stroked on the head or back, and stimulation of the pelvic region induces lordosis (Farris, 1963). Copulation is permitted only at this time. Oestrous is characterized by multiple maturation of ovarian follicles, uterine enlargement and vascularization, and rapid proliferation of the vaginal mucosa resulting in the exfoliation of cornified squamous cells. Oestrous lasts 10-24 hours and ovulation occurs during this period.

Metoestrus-intermediate stage between oestrus and dioestrus. It occurs shortly

after ovulation. Estrogen levels reduce drastically while progesterone levels begin to rise. Metoestrous normally lasts 10-14 hours. At this time the ovaries contain corpora lutea and small follicles and the uteri have diminished in size and vascularity. Many leukocytes appear in the vaginal smears, along with some cornified cells.

Dioestrus-This is characterized by vaginal smears almost entirely containing leukocytes, regression of corpora lutea and the endometrium. Estrogen levels are at their lowest while progesterone levels are at their highest. Dioestrus lasts approximately 48-70 hours.

Prooestrus- is characterized by preovulatory swelling of ovarian follicles; functional involution of the corpora lutea, vaginal smears made up nearly entirely of nucleated epithelial cells. Prooestrus lasts approximately 12 hours.

Female sex steroid hormone biosynthesis and secretion.

It is imperative to understand the nature of the reproductive system and its processes if an attempt to elucidate the possible mode of action of an exogenous substance is to be made.

The keynote in ovarian function is production of viable and fertilizable ova. Failure to do so usually results in infertility. Ova production in most mammals is an episodic event governed by well controlled and well timed cyclic elaboration

of gonadotropins (luteinizing hormone and follicle stimulating hormone) into the blood. These gonadotropins dictate the occurrence of the ovarian cycles which normally consist of the following phases:

(a) follicular growth and development;

(b) ovulation and;

(c) corpus luteum formation.

The basic tenet of reproductive endocrinology include the principle that control of pre-ovulatory follicular growth and development arises from the pituitary gland (Van de Weiele *et al.,* 1970; Ross, 1974; Ross *et al.,* 1979). The pituitary gland produces luteinizing hormone (LH) and follicle stimulating hormone (FSH), which are ascribed roles in initiating and maintenance of follicular growth and selection of the dominant follicle or follicles up to their maturation to pre-ovulatory status. Evidence of this is derived from three observations; the changing patterns of LH and FSH in serum, concomitant alteration in the number and types of gonadotropin receptors in follicular cells, and the secretion of steroidal and non-steroidal products by follicular cells in response to gonadotropin stimulation (Sharon and Geres, 1989; Dizerega and Hodgen, 1981; Goodman *et al.,* 1979; Jones, 1980). The effect of gonadotropins is mediated via plasma membrane receptors located on the cell surface of follicular cells. These receptors respond to gonadotropin binding by activation of adenylate and/or increase in diacylglycerol, production of intracellular messengers, alterations in steroidal enzyme activities and a generalized enhancement of protein synthesis (Lindner *et al.,* 1977; Richards *et al.,* 1983)

The dynamic events of follicular growth may not be accounted for solely by the changing levels of gonadotropins (Goodman *et al,* 1979; Stoifer *et al.,* 1981), rather the development of responsivity of follicles to stimulation by gonadotropins may result from changes in the production of, and alteration in, follicular sensitivity to intraovarian paracrine and/or autocrine factors (Dizerega *et al.,* 1983; Chris *et al.,* 1981; Cahill and Findlay, 1984; Cahill *et al.,* 1985; Chris *et al.,* 1985; Chamming *et al.,* 1985). Intraovarian factors modulate the number and development of ovulatory follicles primarily through regulation of follicular response to gonadotropins (Van de Weiele *et al.,* 1970; Dizerega *et al.,* 1980). In this context, gonadotropins are necessary but insufficient by themselves to account for the ovarian cycle (Tonetta and Dizerega, 1986).

Steroid hormones produced as a result of gonadotropin stimulation of follicular cells include estrogens, progesterone and androgens. During early follicular development, FSH binds to granulosa cells of primary follicles to stimulate production of estradiol by the induction or enhancement of aromatase synthetase (Erickson and Hsueh, 1978; Erickson *et al.,* 1979; Amstrong and Papkoff, 1976). Estradiol in turn induces proliferation of granulosa cells (Rao *et al.,* 1978; Penchanz, 1940; Williams, 1944; Williams, 1945; Dewitt, 1953; Pparyne and Hell baum, 1955; Bradbury, 1961) and increases the sensitivity of the follicles to further gonadotropin stimulation (Ross, 1974; Penchanz, 1940; Simpson *et al.,* 1941; Major and Bradbury, 1960; Goldenberg *et al.,* 1972;

Goldenberg *et al.,* 1973; Johnson and Cheng, 1978). Estradiol can then synergize with gonadotropins to increase ovarian weight, enhance proliferation of granulosa cells and promote growth of pre-antral follicles and antrum formation (Simpson *et al.,* 1941; Goldenberg *et al.,* 1972; Richards, 1975; Lonvet *et al,* 1975; Reiter *et al.,* 1972). In addition, estradiol enhances the responsiveness of granulosa cells to LH and FSH by increasing synthesis of progesterone (Richards *et al.,* 1979; Welsh *et al.,* 1983). The generalized enhancement of gonadotropin action by estradiol is partially mediated by FSH induced accumulation of cAMP (Ingraham, 1954; Ingraham, 1959). Estradiol not only enhances gonadotropin stimulation of LH and FSH receptors in granulosa cells (Ireland and Richards, 1978; Goldenberg *et al.,* 1973), but is required for FSH induction of FSH receptors (Tonetta and Ireland, 1954; Tonetta *et al.,* 1985).

Progesterone and androgens also have intra-follicular effects on follicular growth and steroidogenesis. The presence of progesterone receptors, along with high levels of intrafollicular progesterone, suggest a role for this hormone in follicular development (Jacobs *et al,* 1980; Jacobs and Smith, 1980; Pasqualina and Nguyini, 1980; Philbert *et al.,* 1977; Schrieber and Erickson, 1979).

Androgens augment FSH-induced aromatase activity in granulosa cells, thereby enhancing estrogen synthesis (Daniel and Amstrong, 1980; Hiller and Dezmart, 1981). Androgens can also stimulate the biosynthesis of progesterone in granulosa cells (Schowberg *et al.,* 1978; Leung *et al.,* 1979). FSH and androgens can increase responsiveness of rat granulosa cells to LH as evidenced by

increased production of progesterone and cAMP (Goff *et al.*, 1979). Therefore, androgens act as intraovarian regulators of follicular steroidogenesis.

All steroid hormones are synthesized from cholesterol. The synthesis of progesterone from cholesterol proceeds in the same pathway in the adrenals, the ovaries and the testis. Cholesterol is sourced from plasma low density lipoproteins (LDL) or high density lipoproteins (HDL) as exemplified in rats and humans respectively (Anderson and Dietschy, 1978; Brown *et al.*, 1979; Gwyne and Strauss, 1982). Steroidogenic tissues may, however, synthesize cholesterol *denovo* from acetate (Hetcher *et al,* 1953). Cholesterol produced either *denovo* or sourced from plasma fluxes across the mitochondriol membranes to the cholesterol side chain cleavage enzyme (P450scc). This flux is apparently stimulated by a steroidogenic activator peptide, a 30-amino acid peptide (Pederson and Brommie, 1987) derived from the carboxyl terminus of glucose regulatory protein (Ting and Lee, 1988).

Conversion of cholesterol to pregnenolone in mitochondria (Halkerson *et al.*, 1961) is the first rate limiting and hormonally regulated step in the synthesis of all steroid hormones (Koritz and Kuma, 1970; Jefcoate *et al.*, 1974). This involves three distinct chemical reactions, 20α-hydroxylation, 22-hydroxylation, and scission of the cholesterol side chain at the bond between carbon atoms number 20 and 22 to yield pregnenolone and isocaproic acid (Ommra *et al.*, 1965; Shikita and Hall, 1973). Once pregnenolone is produced from cholesterol,

it may undergo one or two conversions; it may undergo 17α-hydroxylation to 17-hydroxypregnenolone or it may be converted to progesterone, the first biologically important steroid in the pathway. Both pregnenolone and progesterone may undergo 17α-hydroxylation to 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, respectively. These later 17 hydroxylated steroids may then undergo scission of the c-17, 20 carbon bond to yield dehydroepiandrosterone (DHEA) and androstenedione, respectively. This P450c17 is bound to smooth endoplasmic reticulum, where it accepts an electron from a flavoprotein. Androstendione is converted to testosterone, DHEA is converted to androstendiol, and estrone is converted to estradiol by 17-oxidoreductase, also termed 17-ketosteroid reductase, and 17ß-hydroxysteroid dehydrogenase. The enzyme appears to be monomeric, NADPH-dependent, non P450 enzyme bound to the endoplasmic reticulum (Engel and Growman, 1974; Samuels et al, 1975; Inano and Tamaoki, 1974). The aromatization of C-18 estrogenic steroids from C-19 androgens is mediated by P450aro, found in the endoplasmic reticulum. P450aromatase converts androgens to estrogens by two hydroxylations at the C-19 methyl group and a third hydroxylation at C-2, resulting in the loss of C-19 and consequent aromatization of the "A" ring of the steroid (Goto and Fishman, 1977; Fishman and Goto, 1981)

The majority of steroidogenic enzymes are members of the cytochrome P450 group of oxidases (Miller and Levine, 1987; Nebert and Gonzalez, 1987) most of which consist of about 500 amino acids and contain a single heme group. All members of the cytochrome P450 group of enzymes reduce atmospheric oxygen

with electrons from Nicotinamide Adenosine Di-Phosphate (NADPH). $P450_{scc}$ is the cholesterol side-chain cleavage enzyme which mediates the series of reactions 20, 22 desmolase. $P450_{c\ 17}$, found in the endoplasmic reticulum, mediates both 17-hydroxylase and 17, 20-lyase activities. $P450_{\ aromatase}$ in the endolplasmic reticulum mediates aromatization of androgens to estrogens. Intact membranes of the mitochondria and the endoplasmic reticulum are required for steroidogenesis (Sybert *et al.*, 1979).

Signal transduction for gonadotropin hormone action.

Increased intracellular formation of 3′, 5′-AMP (cyclic AMP) is thought to be a general mechanism by which a variety of protein hormones produce their biochemical effects. These hormones include FSH, LH, hCG, TSH, ACTH, insulin, vasopressin and glucagon. Binding of these hormones to the specific receptors on the plasma membrane of target cells causes the activation of adenylate cyclase, an enzyme located on the inner surface of the plasma membrane (Roth and Grunfeild, 1985; Dohlman *et al.*, 1987). A great deal of work has implicated cAMP as the intracellular mediator (or second messenger) of many actions of FSH and LH in the ovary and testis (Gilman, 1987; Litoch, 1987). Evidence that is consistent with this role in gonadal preparations falls into three categories:

I. The addition of hormone to the target tissue (or cells) results in a rapid stimulation of cAMP production, which precedes increases in the end-response.

II. The addition of cAMP (or a derivative such as dibutyrl 3′, 5′-AMP) to the tissue in vitro will mimic the effect of the hormone on the end-response.

III. Agents like theophyline and methyl isobutyl xanthine which inhibit phospodiesterase, will also enhance the end-response.

The many diverse responses produced by cAMP in different tissues may involve the activation of protein kinases. Cyclic AMP-dependent protein kinases are composed of two subunits, regulatory sub-units (R) and catalytic sub-units (C). The binding of the regulatory sub-units to the catalytic components gives an essentially inactive enzyme (Birn *et al,* 1970). Cyclic AMP promotes the dissociation to yield a regulatory (sub-unit + 3′, 5′ AMP) complex and a free enzymatically active catalytic sub-unit. There is a wide distribution of cAMP-dependent protein kinase in mammalian tissues. Protein kinases transfer the phosphate from ATP to a substrate and consequently by this process of phosporylation, the enzymes activate proteins important in the control of cell function (Barber and Butcher, 1980; Gilman, 1987; Nagayama *et al.*, 1989; Spiegel, 1987).

Mechanism of FSH action.

The following sequence of events are said to be involved in the mechanism of action of FSH: FSH binds to its target cells leading to the activation of adenylate cyclase, which results in increases in the intracellular concentration of cAMP. Cyclic AMP then activates protein kinases resulting in the phosphorylation of proteins, which are essential links in the chain of events. The phosphorylated

proteins may bring about their effects in three main ways:

1. By influencing the transcription of DNA, and thus synthesis of specific messenger RNA, which code for proteins required for the expression of FSH receptors;

2. By influencing cell function at the translational level, and thus protein sythesis; or

3. By acting directly on the rate-limiting steps in the reactions involved

Of the known effects of FSH on cellular function, the stimulatory action on estradiol synthesis has been studied in greater detail. Cyclic AMP derivative could produce the same effects as FSH, both quantitatively and qualitatively (Marco *et al.*, 1991; Mc Farland *et al.*, 1989)

Mechanism of LH action.

Of the effects of gonadotropins on the ovary and the testis, the stimulation of steroidogenesis by LH has been studied more thoroughly, but even so its mechanism is far from being well understood. LH accelerates steroidogenesis in its target tissues by influencing the conversion of cholesterol to pregnenolone, which is the rate limiting step in the biosynthetic pathway. LH also increases the levels of cAMP in gonadal tissues rapidly and this effect precedes the increase of steroidogenesis. (Mc Farland *et al.*, 1989)

Control of ovarian function

The control of ovarian function emanates from the medial hypothalamus where the decapeptide hormone, Gonadotropin Releasing Hormone (GnRH), is synthesized and stored in neuro-secretory cells. GnRH is released from neuro-secretory cells in a pulsatile manner into the hypophysio-hypothalamic portal circulation and is transported to the anterior pituitary gland; here it regulates the release of gonadotropins, LH and FSH, into the systemic circulation, also in a pulsatile manner, Conn *et al.*, 1987. Thus, GnRH acts as the key integrator between the neural and endocrine components of reproductive function. The first step in GnRH action (Conn, 1986; Conn *et al.*, 1981;) is its recognition by specific binding sites (receptors) at the surface of gonadotrophs (Marrian and Conn, 1983). It has been demonstrated that GnRH receptors are glycoproteins which contain sialic acid residues (Hazum, 1982) and that the oligosaccharide portion is essential for the function of the receptor as well as for the expression of the receptors on the cell surface of the gonadotropin (Schwartz and Hazum, 1985). Models for GnRH interaction with the receptor have been postulated based on the spatial conformation of the native hormone in solution (Mommany, 1976; Shinitzky and Fridkin, 1976). A consensus is that the driving force for the formation of the hormone-receptor complex is probably an ionic interaction between the amino acid arginine in position 8, which is positively charged , and the negatively charged carboxyl groups in the binding site. In addition to the ionic interaction, the hormone receptor complex may be stabilized by aromatic p-p (pie-pie) interactions between the histidine, tryptophan, and tyrosine

residues of the hormone and the tyrosine and tryptophan residues in the binding site (Mommany, 1976). GnRH binds exclusively to gonadotrophs.

GnRH receptors are initially distributed evenly on the cell surface and then form clusters, which subsequently become internalized (Childs *et al.*, 1983). The latter workers also report that after GnRH receptor-mediated endocytosis, there is substantial degradation of the hormone-receptor complexes within the cell. Internalized GnRH receptors are found in several subcelluler compartments i.e. endosomes, lysosomes, newly formed and mature secretory granules, as well as Golgi cisternae. There are two possible mechanisms that could mediate GnRH action (Hazum, 1982). In the first, the bonding of ligand to receptors leads to cross-linking of receptors in the cell surface which is by itself sufficient to trigger the subsequent biochemical events of hormone action. In the second possibility, however, internalization and degradation of hormone-receptor complexes are important for biological activity. GnRH is one of a family of hormones and neurotransmitters which require calcium for their biological actions (Snuli and Geschmind, 1968). The elevation of intracellular calcium delivered in part via GnRH-activated plasma membrane calcium channels is an important signal for the stimulation of gonadotropin release by GnRH (Clapper and Conn, 1985; Leong *et al.*, 1986; Chang *et al.*, 1986). One probable intracellular mediator of the calcium signal in gonadotrophs is calmodulin, which, after binding calcium alters the activity of several enzymes and proteins implicated in the secretory process (Chafouleas *et al.*, 1982).

1.3.2.0. Research on embelin on non-reproductive parameters.

Many naturally occurring substances with therapeutic properties in humans or animals are discovered accidentally, or through trial and error based on observation in animals. This posses many dangers especially with regard to health risks. While the natural product may prove to be effective as a remedy for some specific ailments, it may have deleterious effects on other systems of the body. Contemporary medical ethics dictate that new natural products (as well as synthetic ones) potentially meant for human consumption be subjected to vigorous efficacy and safety tests before they can be formally accepted as therapeutic agents. An important aspect that must be considered for a new therapeutic product is its mode of action. Knowing its mode of action in the body will help in understanding how the remedial effects are brought about, and also gives an insight as to its potential toxicity in animal tissues.

Numerous qualities have been attributed to embelin as a herbal medicine. Scientists have followed this up with laboratory tests to verify the validity of the observed properties ascribed to it and also to investigate its toxic potential. Some of the work done on this chemical is described below.

1.3.2.1. Pharmacokinetics.

Studies on tissue distribution of embelin have been performed, including determination of the best route of administration. The pharmacokinetics of oral and intravenous administration of potassium embelate (20 mg/kg body weight) was studied in rats (Zutshiu *et al.*, 1990). The results revealed that the compound followed a biexponential kinetic pattern. Absorption was complete (bio-availability was 97%) and fast. The deposition half life was found to be 9.5 hours on intravenous injection and 11 hours on oral administration. High concentrations of the drug were found in the brain between 0.25 and 2 hours, which, according to the workers was in agreement with its pharmacological action. The kidneys were found to play a major role in the excretion of the drug.

1.3.2.2. Toxicity

In their toxicity studies of embelin in laboratory animals (mice, rats and monkeys), Johri *et al.*, (1990) did not find any adverse effects attributed to embelin. Toxicity evaluations included subacute and chronic toxicity testing as well as teratogenic investigations using potassium embelate (2,5-dihydroxy, 3-undecyl-1,4 benzoquinone). These results suggested that potassium embelate was a safe compound. Other workers, however, have obtained results to the contrary. Reports that embelin could possibly be the cause of visual defects among the Ethiopian population prompted Low *et al.*, (1985) to investigate this in laboratory animals. In their study, they found retinal pathology and defects in

visual behaviour in chicks treated with *embelia ribes* and *Hagina abyssinica*, a plant with compounds related to embelin. The extent of deficit in visually guided tasks was found to be dose dependent.

1.3.2.3. Central nervous system.

The analgesic properties attributed to embelin led Johri *et al.*, (1991) to examin how this compound is distributed in rat brain tissues. Radio labeled 3-[H] embelate (a potassium salt of 2,5-dihydroxy-3-undecyl 1,4-benzoquinone) showed a specific and saturable binding in rat brain synaptoses. This was found not to be influenced by naloxone or morphine. The binding characteristics were said to correlate with its non-narcotic central analgesic action.

1.3.2.4. Digestive system.

The effects of embelin on absorptive and digestive functions of rat intestines was investigated by Gupta *et al.*, (1991). They found that oral administration of embelin (75mg/kg bwt. Per day) daily for 15 and 30 days to male rats caused significant elevation in the uptake of D-glucose, L-alanine and calcium in small intestinal segments. Embelin was also shown to cause significant increases in brush border membrane associated enzymes (sucrase, lactase, maltase, alkaline-phosphatase and leucine amino peptidase) in both intestinal homogenates and partially purified brush border membrane preparations. Significant increases were also noted for microsomal glucose-6-phosphatase and cytosolic lactase dehydrogenase. Increase in brush border membrane-associated total lipids,

phospholipids, cholesterol, triacylglycerol, unesterified fatty acids and ganglioside sialic acid were seen but not in the cholesterol/phospholipid molar ratio. All these changes returned to control levels following withdrawal.

1.3.2.5. Effects on mitochondria.

The interaction of embelin with mitochondrial oxidative phosphorylation was investigate by Makawiti *et al.*, (1990). Studies with 4 benzoquinones, namely, jugulone, embelin, maesaquinone and maesanin, on rat liver mitochondria oxidative phosphorylation were carried out. Three of the benzoquinones were found to be uncouplers in the order-Jugulone greater than maesaquinone greater than embelin. Maesanin was found to be an inhibitor of electron transport and oxidative phosphorylation.

1.3.2.6. Anti-tumor and anti-inflammatory properties of embelin.

Chitra *et al.*, (1994) investigated anti-tumor activities of embelin in methylchloranthrane-induced fibrosarcoma in albino rats. It was found that embelin reduced tumor growth in these cells and also enhanced the rats' survival time. The drug was also found to have an appreciable reduction on pain and inflammation. The changes in DNA, RNA and protein levels in various organs in the tumor-bearing controls and the drug-treated animals were found to be significantly different.

In other experiments, Chitra *et al.*, (1995) investigated 3-[H]-thymidine uptake and lipid peroxidation by tumor cells in the presence of embelin in vitro. Using a

rapid technique to assess drug induced cell toxicity, a fibrosarcoma cell line was exposed in vitro to increasing concentrations of embelin and simulteneously inoculated with 3-[H] thymidine. After regular time intervals, the cells were examined for incorporation of the labeled thymidine in DNA, lipid peroxidase and glutathione levels. A dose-dependent decrease in labeled thymidine uptake, lipid peroxidase and glutathione levels was observed on embelin treatment, indicating that the drug had some anti-tumor properties.

1.3.3.0. State of the art research on the effects of embelin on the reproductive system.

Studies have been conducted to evaluate the effects of embelin on the reproductive system, particularly in male laboratory animals. These studies have shown that embelin adversely affects the reproductive system in most laboratory animals. It has been shown to adversely affect testicular histology, reduce gametogenic counts and to lower accessory sex gland fructose levels (Gupta *et al.*, 1990; Jayaraman *et al.*, 1977). Embelin has also been shown to affect male sex steroid hormone biosynthesis and secretion as evidenced by lowered levels of testesterone after embelin exposure in male laboratory animals (Mungai *et al.*, 1997). These changes were reversible.

In female laboratory rats, embelin has been shown to exhibit anti-fertility properties by causing fetal resorption and reducing ovarian weights (Bargava and Dixit, 1985). It has not been shown, however, how embelin affects other

important reproductive parameters such as sex steroid hormone profiles, the evolution of the estrus cycles and how it affects ovarian morphology. It is important to understand how these parameters are affected in order to understand the above observed changes.

CHAPTER TWO

2.0. MATERIALS AND METHODS

2.1.0 Experiment 1. *In-Vivo* Study.

2.1.1. Place, time and animal of study.

This study was conducted at The College of Biological and Physical Sciences (CBPS), of the University of Nairobi in The Department of Animal Physiology, Reproductive Biology Unit (RBU), Chiromo Campus. The study commenced in September 1999 and ended in January 2000.

The animals used were mature (between 3 and 4 months old) female Sprague-Dawley rats which were obtained from the Department of Zoology of The University of Nairobi. All the animals were housed in wire cages of dimensions (45cm x 28cm x21cm). They were provided with fresh water and food in the form of rat pellets (Unga Kenya LTD, Nairobi) ad libitum. The beddings in the cages consisted of wood shavings which were changed daily. The animals were subjected to natural lighting conditions of 12:12 hr light:darkness, and average room temperatures of about 23°c

2.1.2. Chemicals and reagents

Reagents for radioimmunoassay (RIA) were obtained from the World Health Organisation Matched Reagent Programme. These reagents are of human origin, but they have been validated for measurement of hormones in rats in the same

laboratory. The chemical of study (embelin) was obtained from the Department of Chemistry (UoN) where it is extracted and its purity (100%) established by spectrometry (Midiwo *et al.*, 1988).

2.1.3. Methodology

A total of 30 animals were used in this study. Upon arrival in the animal house, the animals were randomly allocated to three groups consisting of 10 animals each. The groups were designated I, II and III based on random allocation of the treatments to each group. Each animal was given an identification mark on its tail. They were then allowed a one week acclimatization period. After the period of acclimatization, vaginal smears were taken from all the animals for 10 consecutive days before commencement of the treatments. This was done so as to ascertain if all the animals were cycling normally. After verifying that they were all cycling normally, they were given another four days rest period before treatments could be effected.

Group I constituted the control group which received subcutaneous injections of corn oil at 1 ml/kg body weight on the flanks every other day for 20 days. Groups II and III were the experimental groups which received subcutaneous injections of embelin dissolved in corn oil at 10 mg/kg body weight and 20 mg/kg body weight, respectively, in a maximum of 0.25ml of corn oil (Oduma *et al,* 1998) every other day for 20 days. All the animals received a total of 10 injections by the end of the experiment. Meanwhile, vaginal swab collection continued on a daily basis throughout the experimental period up to sacrifice.

2.1.4.0. Sample Collection

The following samples were collected from all the animals:

2.1.4.1. Vaginal Smears.

These were collected using clean cotton swabs. Each swab was first soaked in normal saline and gently inserted into the vagina without touching the cervix in order to prevent ovulation induction and hence pseudopregnancy. Upon insertion into the vagina, the swab was twisted slightly and gently and then withdrawn. A smear was then made on a clean glass slide, air dried and then observed under a light microscope at 10x70 magnification. Based on the vaginal swab characteristics, the animals were classified as either being in oestrus, metoestrus, dioestrus or prooestrus.

2.1.4.2. Blood

The animals were first lightly anaesthetised with ether and then bled by cardiac puncture at the end of the experimental period. Blood samples were collected into tubes containing EDTA. The blood was centrifuged at 1500 rpm. Plasma was collected and stored at –20°c until used.

2.1.4.3. Ovaries.

The animals were sacrificed and their abdomens were opened, exposing the intestines. The ovaries were located by following the reproductive tract from the cervix through the bifurcation of the uterine horns to the fallopian tubes.

They were then dissected from their attachments (trimmed as required) and fixed by immersion in 5% formalin until the time of processing for light microscopic examination.

2.1.4.4. Others. The other samples collected included the liver and the pituitary glands. These were also stored in 5% formalin until processing for histopathology.

2.1.5.0. Sample Analysis

2.1.5.1. Progesterone and estradiol determination by radioimmunoassay (RIA).

Progesterone and estradiol levels were determined by RadioImmunoAssay (RIA). RIA is a competitive binding analytical technique whose principle is based on one molecule (the antibody) recognizing another (the antigen) by virtue of the two molecules being congruent in some components of their shapes. In practice, two antigens are made to compete for a limited number of binding sites on the antibody. One of the antigens is labeled with a radio active isotope and is used as a tracer to indirectly measure the unknown quantity of antigen in a sample. The procedure used in the assay of the two hormones in this study is as that outlined in the World Health Organization RIA manual of 1993. Briefly, some of the critical steps followed are given below:

The reagents provided for both progesterone and estardiol included antisera,

standards, tracers, charcoal reagents, gelatin and dextran. The antisera and tracers are for human samples, but they have been validated for laboratory rats in the same laboratory (RBU).

Standards were prepared as follows:

The residue from 100µl of ethanolic solution was reconstituted with 10ml of buffered saline. This was mixed gently and heated to 40°c for 30 minutes. It was then mixed vigorously and the solution allowed to cool to 4 degrees centigrades before use. These solutions thus finally contained 1.5 nmol/l and 2.5 nmol/l of estradiol and progesterone respectively. These solutions were then serially diluted in 6 labeled test tubes (for each hormone). From these serially diluted solutions, triplicate 500µl aliquot were pippeted directly into assay tubes.

Samples were prepared as follows:

First, steroid hormones in the plasma samples were extracted. This was done by pipetting 400µl aliquots of plasma and transferring to labeled tubes with screw on caps. About 4mls of diethyl ether was added to each tube and the tubes tightly covered. The tubes were then mounted on to a shaker were extraction was done for at least 5 minutes. After extraction, the aqueous and non-aqueous phases were separated by placing the tubes in a cold water bath. The ether (together with steroid hormones) was decanted into labeled tubes and evaporated in a Brinkman/Buchler vacuum drier. After evaporation, 2ml of buffered saline was added to each tube and carefully vortexed. This solution was then left for 10 minutes and vortexed again. Triplicate 500ul of these solutions was then pippeted into assay tubes.

All in all, the assay finally contained the following tubes:

Total count tubes (TCs); Non specific binding tubes (NSBs); Zero antigen tubes (BOs); standard tubes; ether blanks; unknown samples plus three sets of quality control samples-one at the beginning, one in the middle and one at the end of the assay.

Reagents were added as follows:

Total count tubes (TCs)-100µl of the working dilution of 3H-estradiol/progesterone and 800ul of buffer.

Non specific binding tubes (NSBs)-100µl of the working dilution of 3H-estradiol/progesterone and 600µl of buffer.

Zero antigen tubes (BOs)-500µl of buffer, 100µl of tracer, 100µl of antiserum.

Ether blank unknowns-500µl of sample from a vial containing reconstituted ether residues only, 100µl of tracer, 100µl of antiserum.

Standards and unknown Samples-500µl of standard or unknown sample, 100µl tracer, 100µl antiserum.

The tubes were then incubated at 4°c overnight. After incubation, 200µl of cold dextran coated charcoal (0.625g charcoal, 0.0625g dextran in 100ml assay buffer) was addded to each tube except the TCs. The tubes were then vortexed

and left to stand for 15 minutes at 4 degrees centigrades. The tubes were centrifuged at the same temperature for 15 minutes at 1500g using the Beckman model TJ-6 centrifuge connected to a TJ-Refrigeration unit. The supernatant from each tube was decanted into scintillation vials containing 4ml of toluene scintilation fluid. The scintilation fluid was allowed to equilibrate in the dark. Counting was finally done using a Packard PRIAS counter. Results from the samples were calculated from the standard curve.

2.1.5.2. Tissue preparation for light microscopy.

The fixed tissues were removed from the fixative. They were first washed in running tap water overnight to remove excess fixative. After washing, dehydration of the tissues was done by passing them through progressively higher concentrations of ethyl alcohol (50%, 70%, 80%, 90% and finally absolute alcohol, for at least 3 hours in each alcohol concentration). The alcohol was removed from the tissues by immersing them in methylbenzoate. The tissues were then infiltrated with parafin wax and finally embedded in molten paraplast. The tissues were serially sectioned on a microtome, mounted on slides and stained with eosin and heamatoxyline. The slides were covered with cover slips and observation done under a light microscope.

2.1.5.3. Statistical Analysis

One way analysis of variance (one way-ANOVA) was used. The difference between the means was considered significant at $P<0.05$. Values are reported as means ± SEM.

2.1.6. Results

Figures 1, 2, and 3 depict the evolution of the oestrous cycles in the control, and treatment groups (I and II) respectively. Figure 1 shows that all the animals in the corn oil-treated (control group) were having normal 4-5 days oestrous cycles through out the experimental period. Corn oil did not alter or disrupt the evolution of the estrus cycles. On the other hand, some animals in the treatment groups, figure 2 and 3, had disrupted oestrous cycles. Specifically, 3 animals in group II and 4 animals in group III essentially remained in diestrus by the 5^{th} day of injection.

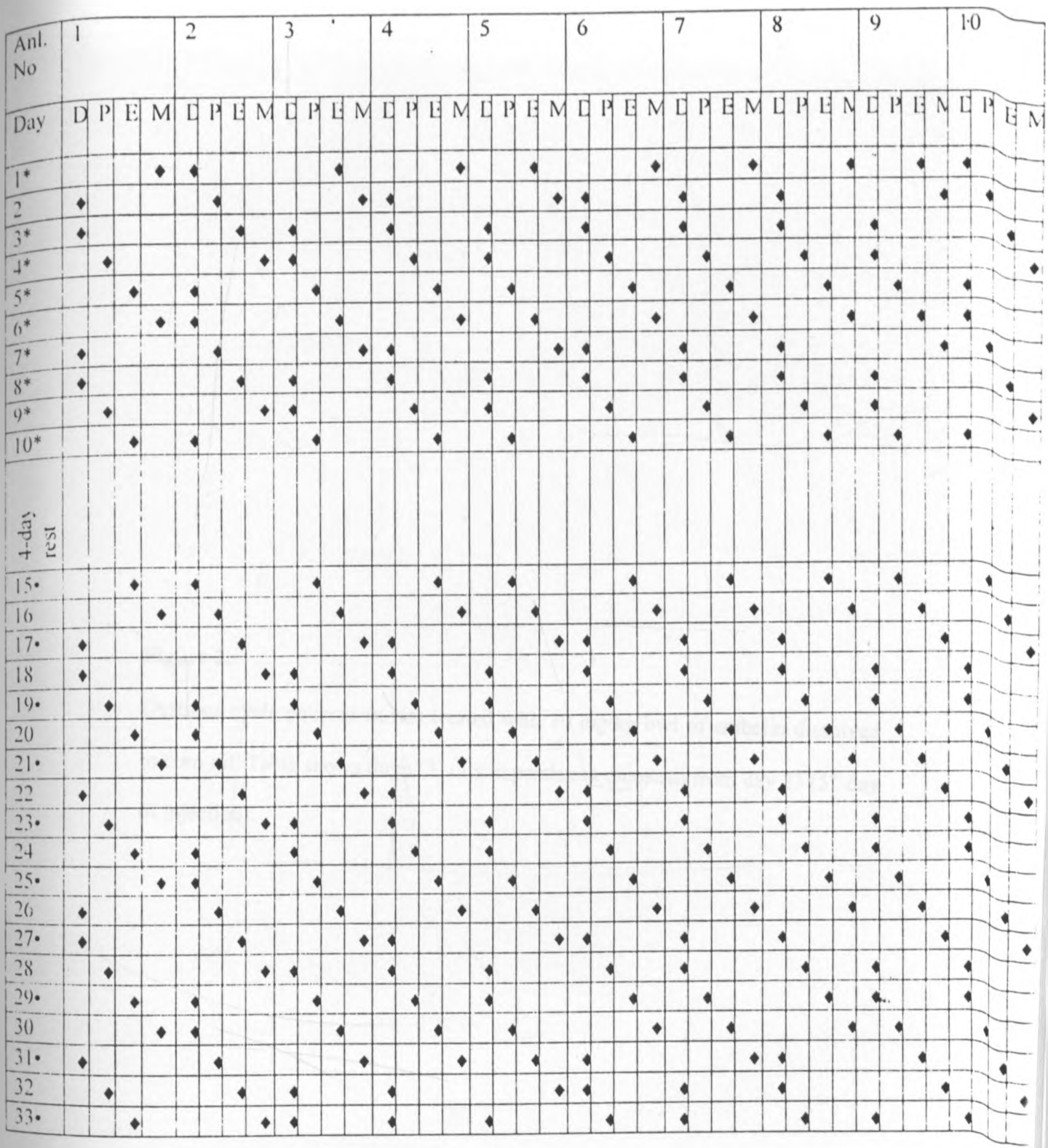
Figure 4 depicts estradiol profiles of the control and the two experimental groups. Plasma estradiol levels in the control animals ranged between 586 ± 37.2 pmol/L at oestrous and 275.4 ± 22 pmol/l at dioestrous. Plamsa estradiol levels in both the experimental groups were significantly different ($p<0.05$) from the control group.

Figure 5 depicts the progesterone profiles of the control and the experimental animals. Plasma progesterone levels in the control animals ranged between 23.30 ± 0.10 nmol/L at dioestous and 5.97 ± 0.32 nmol/L at oestrous.

Plasma progesterone levels at both the dose levels were significantly different ($P< 0.02$) from those of the control group. These results suggest that embelin at both the dose levels used in this experiment suppressed plasma progesterone levels, the dose not withstanding.

Figure 1.

Oestrous cycle patterns in corn oil-treated control rats as monitored by vaginal cytology. Table shows that generally rats exhibited regular 4-5 days cyclical patterns.



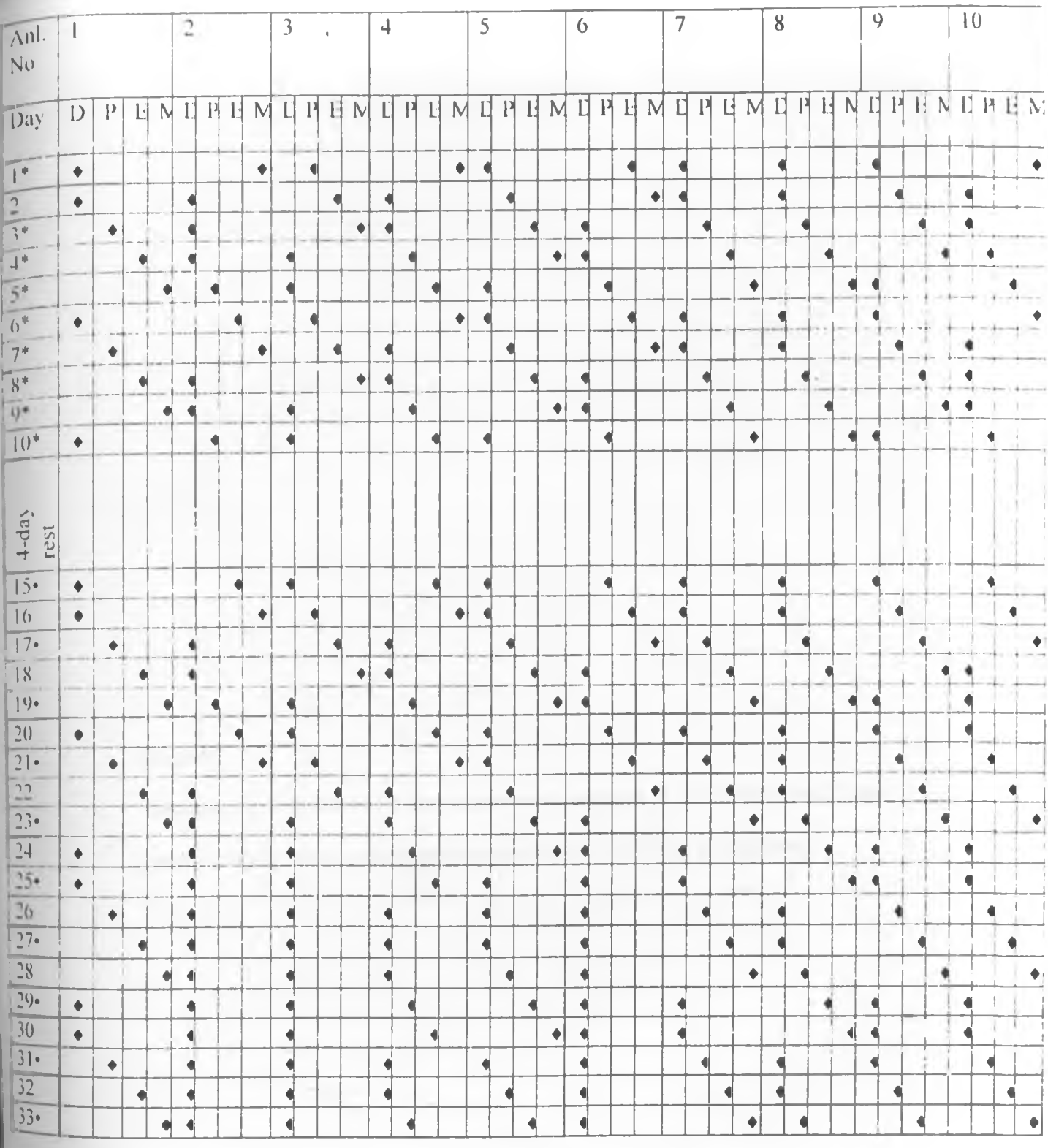
D=Diestrus; P=Proestrus; E=Estrus; M=Metestrus

(*) = Pre-treatment Days (cycle monitoring)

(•) = Days of Injections

Figure 2.

Oestrous cycle patterns in rats treated with 10 mg/kg bwt of embelin dissolved in corn oil. Table shows three (3) rats in persistent dioestrus from day 23 (5^{th} day of injection).



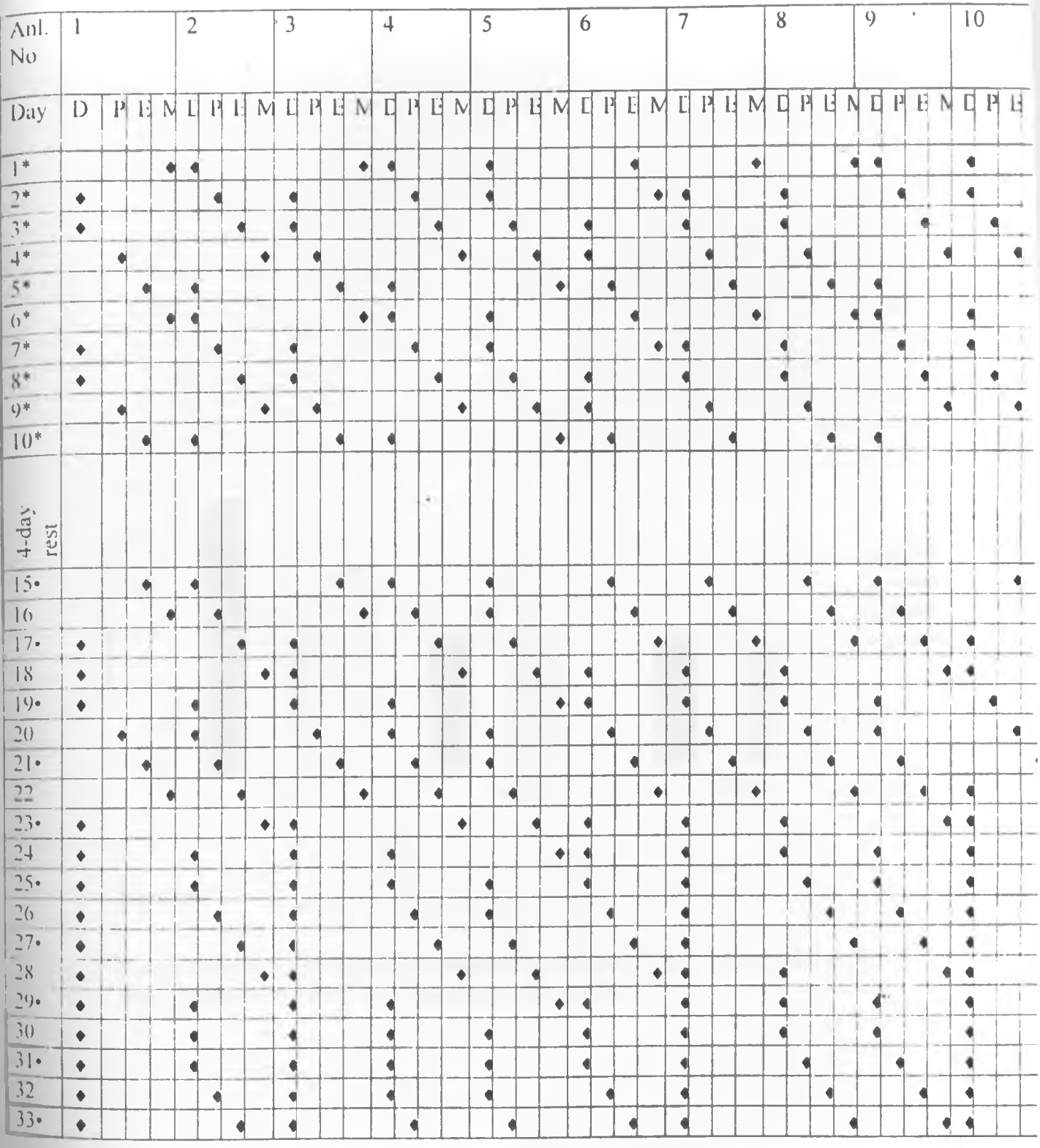
D=Diestrus; P=Proestrus; E=Estrus; M=Metestrus

(*) = Pre-treatment Days (cycle monitoring)

(•) = Days of Injections

Figure 3

Oestrous cycle patterns of rats treated with embelin at 20 mg/kg bwt. Table shows four (4) rats in persistent dioestrus by day 23 (5^{th} day of injection).



D=Diestrus; P=Proestrus; E=Estrus; M=Metestr

(*) = Pre-treatment Days (cycle monitoring)

(•) = Days of Injections

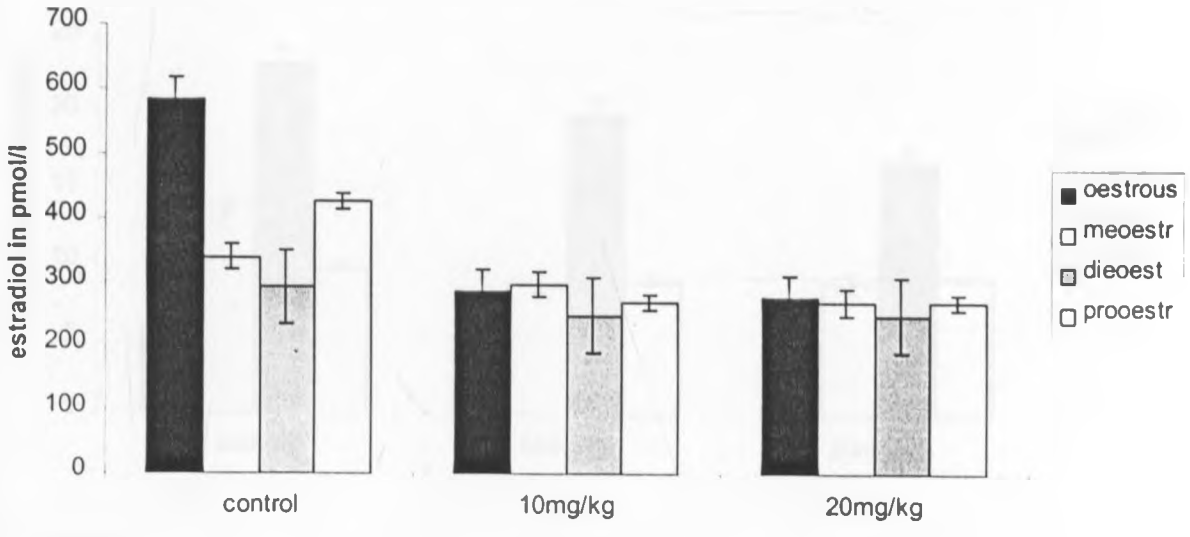


Figure 4

Plasma oestradiol levels in corn 0il-treated rats and rats treated with embelin at 10 mg/kg bwt and 20 mg/kg bwt. Values are means ± SEM

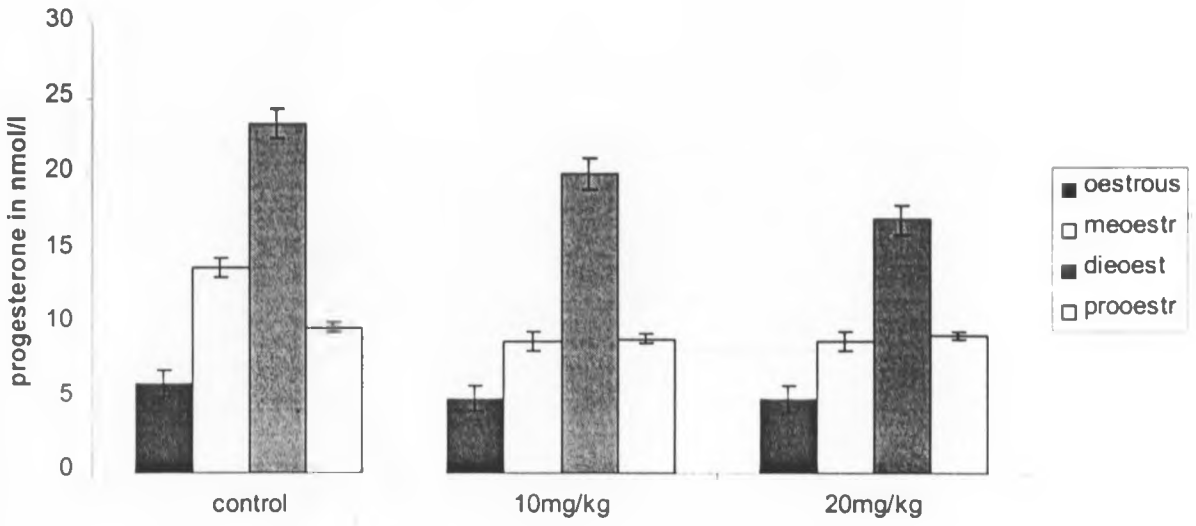


Figure 5

Plasma progesterone levels in corn oil-treated rats and rats treated with embelin at 10 mg/kg bwt. and 20 mg/kg bwt. Values are means ± SEM

Plate 1.

Light micrograph of histological section of ovary showing a normal follicle. Note the circular symmetry of the oocyte (Oo), and intact zona pellucida (big arrow), nucleus (small arrow), granulosa cells (Gr) and developing antrum (A). Eosin and Heamatoxyline (40x)

Plate 2

Light micrograph of histological section of ovary showing a follicle from an embelin treated rat. Note the rough edges in the symmetry of the oocyte (arrow heads). Another follicle (F) is devoid of an oocyte.

Eosin and Heamatoxyline (40x)

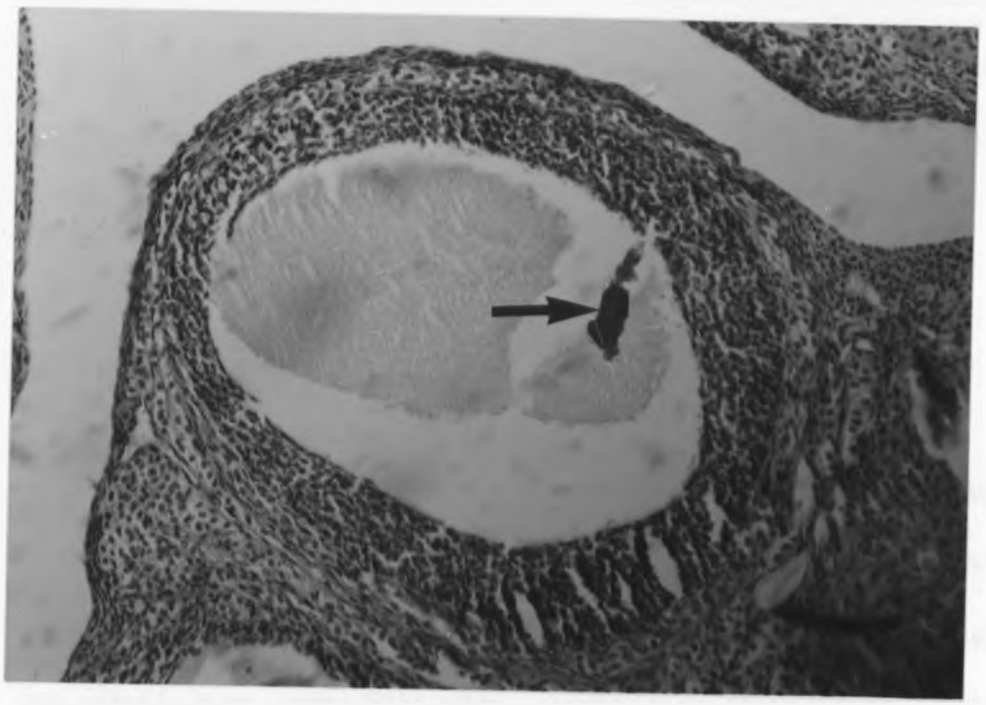


Plate 3

Light micrograph of histological section of ovary showing mature follicle from an embelin treated rat. Note the absence of the oocyte and remnants of zona pellucida (arrow).

Eosin and Heamatoxyline (40x).

2.2.0. Experiment 2-*In-Vitro* Study.

2.2.1. Chemicals and Reagents.

Highly purified pituitary gonadotropins were used in this study. Luteinizing hormone (LH) stock solution had 50IU while Follicle Stimulating Hormone (FSH) stock solution had 40 IU. These reagents (together with Bovine Serum Albumin) were obtained from WHO matched reagent programme courtesy of Reproductive Biology Unit (RBU) of the Department. Tissue culture medium - Minimum Essential Medium (MEM) modified with Earl's salts was used as the basic medium. This was obtained from Flow Labs, Irvin U.K.

2.2.2. Cell preparation for *In-Vitro* study.

A modified mouse interstitial cell bioassay was adapted in this study.

Four petri dishes containing chilled (4° C) and aerated (CO_2 5%:O_2 95%) MEM were prepared prior to killing the rats. Three mature (3-4 months old), normal cyclic female Spraguey-Dawley rats were used. Ovaries were collected while the animals were under light ether anaesthesia as explained previously in experiment 1. The ovaries were transfered to the first petri dish. Here, they were trimmed of excess tissue and transfered to the second petri dish where they were cut into at least four pieces each with a sharp scalpel blade. They were washed thoroughly in this petri dish before being transferred to the third petri dish where a final wash was done. Finally, the ovarian pieces were transferred to the fourth dish containing the same medium but containing also 0.1% collagenase to facilitate dispersion of the cells. The ovaries had to undergo

several washings in this manner in order to eliminate as many erythrocytes as possible from them. In the fourth petri dish, they were finely minced with scissors. The cell mixture was then filtered through glass wool, the debris discarded and the cell suspension pre-incubated in plastic flasks for about 30 minutes at 37° C in a shaking water bath. The cell suspension was then centrifuged at 1000 rpm. The supernatant was discarded and the cell pellet re-suspended in MEM containing Bovine Calf Serum and stirred slowly to disperse uniformly. Cell viability counts (see appendix) were then done using tryptan blue. The cells were inoculated at a density of 2.5 x 10^5 live cells per tube in 200µl of medium.

2.2.3. Reagents preparation

The required strengths of gonadotropins (FSH and LH) were constituted. Embelin was prepared by dissolving it in DMSO. Two doses of embelin were prepared; a high dose (4.5 mMols, 2.1 mg) and a low dose (2.25mMols, 1.05 mg).

2.2.4. Experimental procedure

The experiment was finally set up as follows:

(I). Experimental Tubes.

This consisted of tubes in triplicate, to which was added LH (50µl); FSH (50µl); cell suspension in MEM (200µl); and 20µl of the desired doses of embelin in DMSO. The final volume came to 320µl.

(II). Basal Secretion

This consisted of tubes in triplicate to which was added 200µl of cell suspension in MEM plus 120µl of PBS-BSA solution. These tubes were used to determine the basal secretion of the cells in the absence of any reagent.

(III). DMSO Controls.

This also consisted of tubes in triplicate to which was added 200µl of cell suspension, 50µl of LH and 50µ of FSH and 20µl of DMSO solution.

All the tubes were incubated under CO_2:O_2 (5%:95%) gas in a shaking water bath at 37° C. Embelin and DMSO were added first to the cell suspension and given 4 hours of incubation time so that they could be incorporated into the cells. Gonadotropins were then added to the tubes and incubation continued for another 3 hours. Incubation was terminated and the tubes centrifuged to precipitate the cells. The supernatants were collected and used for RIA to determine the levels of progesterone and estradiol secreted by the mixed ovarian cells. These levels, obtained as a result of LH and FSH stimulation in the presence of embelin or DMSO, were converted to % response when compared to those of basal secretion, which was designated 100%. Levels of less than100% were interpreted as negative response or inhibitory while those above 100% were taken as positive response or stimulatory.

2.2.5. Results.

Table one shows the results of the in-vitro studies. From the table it can be seen that embelin suppressed both estradiol and progesterone secretion by ovarian cells in-vitro.

	Basal Secretion	% Response	Embelin at 2.25 mmol.	% Response	Embelin at 4.50 mmol.	% Response
Estradiol in pmol/L	107.23± 3.47	100	84.57± 4.82	78.88	83.56± 2.60	77.92
Progester in nmol/L	4.12± 0.11	100	3.64± 0.13	88.34	3.57± 0.10	86.65

Table 1.

Table shows values of estradiol and progesterone secreted by mixed ovarian cells after direct challenge with embelin in-vitro. The % response in relation to basal secretion is also indicated. Values are means ±SEM.

CHAPTER THREE

3.0.0. DISCUSSION AND CONCLUSIONS

3.1.0. Discussion

3.1.1. The present study

Introduction

This study was set up to investigate the effects of embelin on some reproductive parameters in female Sprague-Dawley rats. Results from this study indicate that embelin interferes with reproductive parameters in this animal model. All the parameters considered in this study were adversely affected; disruption of the normal estrous cycle was accompanied by significantly lower levels of progesterone and estradiol in the experimental animals. The most significant effect was on progesterone. Ovarian morphology was also adversely affected.

In another experiment, isolated ovarian cells were directly challenged with embelin in order to investigate their biosynthetic and secretory capabilities in the presence of embelin. These *in-vitro* studies showed that embelin had a direct effect on isolated ovarian cells.

3.1.2. Effects of embelin on plasma progesterone and estradiol

Data in this study indicate that embelin suppresses both plasma progesterone and estradiol in rats. Plasma progesterone and estradiol levels are usually used as indicators of the integrity of reproductive function. Suppression of plasma sex

steroid hormones after introduction of an exogenous substance into an animal may mean that the exogenous substance is actually a reproductive toxin.

In experiments performed to determine the effects of an exogenous substance on the reproductive system, two groups of laboratory animals are normally used. One is the control group, in which the experimental substance is withheld. Instead a placebo or the vehicle used to dissolve the chemical substance is administered e.g. corn oil for lipid soluble substances like embelin. The second group of animals is the experimental group consisting of usually two or more groups of animals which receive the test drug at different doses. At the end of the experiment plasma progesterone and estradiol levels are compared between the control and the experimental group. Any statistically significant difference in hormonal levels between the two groups of animals means that the experimental drug suppresses (if experimental is lower than control) plasma hormonal levels. However, this does not say how or where the exogenous substance is exerting its effects.

The many processes involved in the production of sex steroid hormones in famale rats means that embelin may be acting at one or more places along the hypothalamus-hypophysial-gonadal axis. Possible points of action for exogenous substances include the hypothalamus, the pituitary gland and the gonads. The biosynthesis and secretion of the decapeptide hormone, gonadotropin releasing hormone (GnRH) in the neuro-secretory cells of the hypothalamus is one of the most fundamental processes required in the control of reproductive function. GnRH

is released into the hypothalamus-hypophysial portal system where it is transported to the hypophysis (Setalo *el al.,* 1975). Within the hypophysis, GnRH controls the release of the two gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) which in turn control the biosynthesis and secretion of the steroid hormones (progesterone and estardiol) in female rats (Zimmerman, 1976). For optimal synthesis of the reproductive hormones, GnRH must be released in a pulsatile manner and at an appropriate amplitude. (Setalo *et al.,* 1975). Failure to do so will affect the biosynthesis and secretion of gonadotropins which will in turn affect sex steroid hormone biosynthesis and secretion. It is possible that embelin may be interfering with either synthesis, pulsatile release or binding of GnRH to gonadotrophs.

Embelin may also directly affect gonadal function within the ovaries or by altering the feed back mechanism to the hypothalamus and pituitary gland. Alterations in the hypothalamus-pituitary portion may in turn disrupt gonadal function. Also at gonadal level, embelin may be interfering with any one of the following processes: binding of gonadotrophs to follicular cell surface receptors resulting in reduced or abolished signal transduction for the biosynthesis of steroid hormones; interference with one or more steroidogenic enzymes within follicular cells. In male laboratory animals (rabbits) embelin has been shown to interfere with testosterone production at testicular level (Githui *et al.,* 1991). These workers reported low testosterone levels concommitant with high levels of luteinizing hormone as compared to controls, indicating that embelin was actually acting at testicular level. It is possible

that embelin could be acting at ovarian level in this study. Unlike in the male, however, LH is not useful in determining if an exogenous substance is acting at ovarian level or not because of its fluctuations through out the oestrous cycle.

Low levels of progesterone and estradiol in embelin treated rats could also be due to increased elimination of the steroid hormones in the liver and kidney, although there is no evidence to show that embelin increases steroid hormone excretion.

Results obtained by Prakash and Vashney, (1985) that embelin treatment results in reductions in ovarian weights and pregnancy losses through fetal resorption can be explained from the results obtained in this study. The phenomenon of fetal resorption reported by these workers may be attributed to progesterone deficiency due to poor luteal function caused by the action of embelin. Progesterone (secreted from corpora lutea in the ovary) is necessary to maintain pregnancy up to term in rats (Sokolowki, 1971; Andersen, 1970).

3.1.3. Effects of embelin on ovarian morphology.

Results obtained in this study indicate that embelin adversely affects ovarian morphology (Plates, 2 and 3). The most notable changes in this study included complete destruction of oocytes with evidence of remnants of zona pellucida in the experimental animals. There were also degeneration of oocytes in the experimental animals as compared to the control animals (Plate 3).

The oocyte is the fundamental element of both the structural and functional integrity of the follicle complex. When the oocyte degenerates, other follicular components either die or "dedifferentiate" and steroid hormone biosynthesis and secretion are changed both quantitatively and qualitatively. Changes in the appearance of the degenerating oocyte have been investigated with the light microscope (Ingram, 1959). About 50-70% of the oocytes in a normal mammalian ovary appear, in the light microscope, to be degenerating, but the percentage cannot be determined precisely because of the subjective element involved in the recognition of degeneration. However, changes such as complete destruction of oocytes and altered oocyte morphology are relatively easy to recognize. In this study ovaries from experimental rats showed many oocytes with altered symmetries in their shape i.e. from smooth circular to rough edged as compared to the controls. Other oocytes were completel destroyed. This is evidence that embelin altered ovarian (oocyte) morphology. Emeblin could be affecting ovarian morphology by acting directly on oocytes or preventing access of essential elements to reach the oocyte.

3.1.3. Effect of embelin on oestrous cycles of rats.

Results in this study indicate that embelin interferes with the normal estrous cycles of rats.

Estradiol suppression by embelin is most likely the cause of the disruption of the evolution of the normal estrus cycles. This is because estradiol is the main hormone responsible for the cyclic transformation of the vaginal epithelium. Places of action and the primary mechanisms of action of estradiol on the

receptor cells have been investigated particularly with respect to RNA, protein synthesis and its binding with cytoplasmic and nuclear proteins in the vagina (Mueller, 1965; Talmar, 1970; Talmar *et al.*, 1968; Jense *et al.*, 1969; Baulieu and Rochefort, 1970). The cornification of the vaginal epithelium during estrus is the result of changes provoked by estrogen at the cell level (Takasugi, 1963). The epithelium of the vagina reacts to the effect of estrogen by stratification and keratinization (Zuckerman and Parkes, 1976). The vaginal epithelium loses its cornification when estrogen has been withdrawn, such as occurs during the diestrous phase of the cycle. Therefore, any substance that suppresses plasma estradiol levels would also cause the vaginal epithelium to involute and assume the diestrus characteristics. This is probably what embelin had done in this study. The estrous cycle in the rat is a good indicator of the patency of reproductive function in general.

3.1.5. Effects of embelin on isolated mixed ovarian cells

Results from Experiment 1 have shown that embelin interferes with the female sex steroid hormone profile in the Spraguey-Dawley rat animal model. This was also accompanied by disruption of the evolution of the normal estrous cycles of the rats (see figures 1 and 2) and some alterations in ovarian morphology (see plates. Simply knowing that embelin affects these reproductive parameters negatively may not be enough. It is important to know (or at least to postulate) at what point within the reproductive system it is exerting its effects. Along the hypothalamus-pituitary-gonadal axis, the gonadal point provides a convenient

point to examine the effects of this compound since it is relatively easy to isolate and culture ovarian cells. This experiment was instituted in order to try and elucidate where along the hypothalmus-pituitary-gonadal axis embelin might be exerting its effects. The ovarian point was not only selected for its convenience, but also because morphological characteristics of ovarian components in the in-vivo study indicated profound alterations after embelin treatment. These changes might have been due to the action of embelin on ovarian cells and so it was imperative that this be verified in vitro. The main aim of the experiment was to investigate the effect of embelin on steroid hormone biosynthetic ability of isolated ovarian cells.

So far no studies have been conducted in which isolated ovarian cells were exposed to embelin and the cells evaluated for any functional changes. There have been, however, some studies in which other cell types were exposed to embelin and certain functional aspects of these cells assesed. For example, Chitra *et al.*, (1995) investigated the uptake of 3[H]-thymidine and lipid peroxidation by fibrosarcoma cells in the presence of embelin. A dose-dependent decrease in labeled thymidine uptake, lipid peroxidation and glutathione levels was observed. Other studies involved the exposure of isolated ovarian cells to other chemical substances followed by assesment of some of their functional aspects. Oduma *et al.*, (1998) exposed isolated ovarian cells to heptachlor (an organochlorine compound) and assesed their sex steroid hormone biosynthetic capability; heptachlor suppressed the cells` ability to synthesize sex steroid hormones in-vitro.

Ovarian cells (and indeed other cell types) are normally cultured in media that are largely aqueous. The culture medium used in this experiment (MEM) is also largely aqueous. Oduma et al. (1998) dissolved heptachlor in very minute quantities of Dimethyl Sulfoxide (DMSO) before introducing it into the medium. DMSO was used to in order to allow the embelin to easily gain access to the cells in the aqueous medium. The quantity of DMSO used was considered to be too minute to cause any confounding effects on the outcome of the experiment. In this study, the same amount of DMSO as used by Oduma, (1998) was also used to disolve embelin. However, DMSO controls were also included here, just in case DMSO's potency is high enough to cause significant effects on the outcome.

Data in this experiment indicated that embelin actually suppressed secretion of progesterone and estradiol in-vitro by isolated ovarian cells. This may imply that embelin actually exerts its effects in the rats at ovarian level.

3.1.6. Possible mode of action of embelin.

The biosynthesis and secretion of mammalian female sex steroid hormones involves a myriad of processes and components within the reproductive system emanating from the hypothalamus through the pituitary gland to the gonads. Each component involved in reproductive function (directly or indirectly) is important. Each can adversely affect hormone production in one way or another if perturbed. To establish precisely where along the hypothalamus-pituitary-

gonadal axis abberative effects are taking place as a result of the action of an exogenous substance is not an easy task. It is possible, therefore, that embelin may be exerting its effects at any point along this axis eventing in the results obtained.

Results from the in-vitro studies in this experiment have indicated that embelin can suppress hormone secretion by these cells. This may mean that even in live animals embelin may be exerting its effects at ovarian level. However, how it is exactly doing this at ovarian level is a matter for speculation at this moment. Perhaps, results obtained by other workers concerning the effects of embelin on some important components of cellular function can shed some light as to the possible mode of action of embelin. Of particular interest is the work done by Makawiti *et al.*, (1990) who examined the effect of embelin on the mitochondrial oxidative phosphorylation. These workers found that embelin (and some other related compounds) uncoupled oxidative phosphorylation in rat liver mitochondria. It is possible that embelin could do the same in other tissues or organs, including the ovary. In section **1.3.1.** it is reviewed that the signal transduction of the message after gonadotropin binding to surface receptors into the cell is achieved through the accumulation of the second messenger, cAMP. Cyclic AMP acts as the intra-cellular amplifier of the signals from gonadotropins after they have interacted with plasma membrane receptors (Gilman,1984; Boney *et al.*, 1983). The role of cAMP within the cell is in the activation of protein kinases. Protein kinases in turn activate essential proteins (e.g. enzymes) involved in critical steps of sex steroid hormone biosynthesis

(Boney et al., 1983). The activation of these essential enzymes by protein kinases is achieved through the process of phosphorylation. Protein kinases acquire phosphate groups used to phosphorylate enzymes from the energy rich ATP which is made in the mitochondrion through the chain of processes termed, oxidative phosphorylation. A compound that uncouples oxidative phosphorylation disengages one of the key points in this chain from the rest of the components. The end result is a reduction in total ATP produced within the cell. This means that protein kinases have little or no ATP to source their phosphates from to use in the activation of essential steroidogenic enzymes. As a result steroidogenesis ceases or is drastically mitigated.

Another point of view to look at it is from cAMP. This compound is also made from the hydroxylation of ATP. If the levels of ATP in the cell dwindle, then few or no cAMP will be produced. Such a scenario will inevitably result in a weak signal transduction or its complete abolition all together. In short, embelin may be suppressing sex steroid hormone biosynthesis by indirectly causing low levels of ATP.

3.1.7. The future

More investigations need to be done in a number of areas in order to completely determine anti-fertility properties in female animals. The most important areas that need to be evaluated include determining the reproductive performance such as litter sizes in laboratory animals after embelin exposure. Also investigations

need to be done to determine the reproductive performances of ofsprings born of animals previously exposed to embelin.

Another area that needs to be considered is the pharmacodynamics of embelin. It is not very clear whether embelin or its metabolites is causing the observed results.

3.2. Conclusions

This study has established the following new information on the effect of embelin on some reproductive parameters of female Spraguey-Dawley rats:

1. Embelin alters the evolution of the normal estrus cycles in this animal model as evidenced by vaginal cytology after embelin exposure.
2. Embelin suppresses plasma progesterone and estradiol levels in this animal model.
3. Embelin exposure also results in morphological changes in the ovary as evidenced by granulosa cell dis-aggregation and changes in the symmetry of oocytes.
4. Embelin acts directly on ovarian cells as evidenced by its suppressive effects on the secretion of progesterone and estradiol by isolated ovarian cells.

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APPENDIX

VIABILITY OF OVARIAN CELLS

The viability of ovarian cells was estimated by the dye dilution method using trypan blue to distinguish between dead and live cells. Live cells appear clear because of their ability to digest the dye, while dead cells appear stained because of their inability to digest the dye. The % viable cells was estimated as follows:

$$\% \text{ viable} = \frac{\text{total viable cells (Unstained)}}{\text{total cells (unstained plus stained)}} \times 100$$

The viability in this experiment was 87%.

Purity of cell preparation

The purity was determined as follows:

$$\% \text{ purity} = \frac{\text{number of mixed ovarian cells} \times 100}{\text{total number of cells}}$$

The purity of ovarian cells was 92%