EFFECTS OF

A SUPEROVULATORY DOSE

OF PREGNANT MARE'S SERUM GONADOTROPIN

ON UTERINE MORPHOLOGY, CARBOHYDRATE HISTOCHEMISTRY

AND SERUM STEROIDS IN IMMATURE RATS

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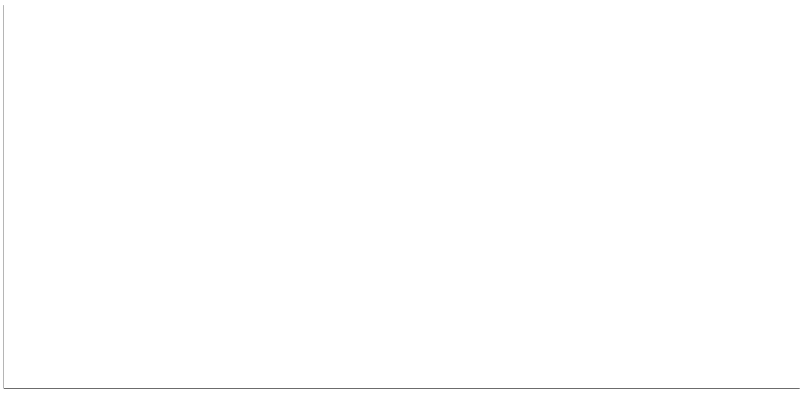

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ABSTRACT

Infertility, defined as the failure to conceive or induce conception, is a common and serious problem in both humans and domestic livestock. Superovulation with exogenous gonadotropins in conjunction with in vitro fertilization and embryo transfer is one method used to treat this problem. However, superovulation is associated with a decreased pregnancy rate. The objective of this research was to determine whether this decreased fertility rate was associated with changes in the uterus. This study compared the effects of a superovulatory (40 IU) and a physiological (4 IU) dose of pregnant mare's serum gonadotropin (PMSG) on the morphology of the endometrium and the carbohydrate composition of the uterine glycocalyx and secretions in immature rats and in untreated mature rats. The morphological and carbohydrate histochemical changes found were correlated to the serum concentrations of estradiol, progesterone and androgens.

Female 28 day old Sprague-Dawley rats were injected with PMSG (4 or 40 IU, subcutaneous). Subgroups of rats (n = 6 /dose) were sacrificed immediately before injection and at 24 h intervals for 9 days after initial treatment (total n = 114). Serial 4 micron thick paraplast sections of Bouin's fixed uteri were stained with hematoxylin and eosin for morphological assessment. A battery of histochemical staining methods selective for sulphate esters, sialic acid, neutral sugars and glycogen were used for assessing the carbohydrate composition of the uterine secretions and the glycocalyx. Serum estradiol, progesterone and androgen concentrations were determined by radioimmunoassay.

The morphology of the endometrium, the amount and carbohydrate composition of the uterine secretions and glycocalyx as well as the steroid patterns in the 4 IU PMSG treated rats were similar to that seen in the mature rats. In the 40 IU PMSG group, the morphological changes of the endometrium include hyperplasia, disorganization of the subepithelial stroma and an increase in the length of the internal lumen perimeter. This

superovulatory group had an increase in the amount of the uterine secretions and glycocalyx when compared to the mature and 4 IU PMSG group ($p<0.001$). The secretions within the superovulatory group showed increases in the amount of sialic acid and neutral sugars present, while the glycocalyx showed increases in only neutral sugars when compared to the mature and 4 IU PMSG group ($p<0.001$). The serum concentrations of estradiol, progesterone and androgens were higher in the superovulatory group ($p<0.05$) and the morphological and histochemical changes found in the uteri of superovulated rats may be caused by these prolonged and increased estradiol and progesterone concentrations. These morphological and endometrial changes may result in a uterus that is no longer suitable for proper blastocyst-endometrial interaction and therefore prevent successful implantation in the superovulated rat.

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LIST OF ABBREVIATIONS

<	less than
=	equal
>	greater than
%	percent
&	and
α	alpha
β	beta
^3H	tritium
AB	alcian blue
AI	artificial insemination
aqua	aquamarine
Bh	borohydride
B_o	maximum binding
C.I.	colour index
C7	carbon seven location
cm	centimetre
cpm	counts per minute
d	day
di	two
die	diestrus
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
ER	embryo replacement
est	estrus
ET	embryo transfer
fsh	follicle stimulating hormone
g	gram
GIFT	gamete intrafallopian transfer
h	hour
H_2O	water
ie.	for example
IGF	insulin-like growth factor
IL-6	Interleukin 6
IU	international units
IVF	in vitro fertilization
KOH	potassium hydroxide
LH	luteinizing hormone
M	mole
met	metestrus
min	minutes
Ml	millilitre
mm	millimetre
Mm	millimole
mRNA	messenger RNA
n	number in sample
NaCl	sodium chloride
ng	nanogram
np	not present

NSB	non-specific binding
p	statistical probability
P/E Ratio	progesterone/estradiol ratio
PA	periodic acid
PA*	selective periodic acid oxidation
PAS	periodic acid schiff
PBS	phosphate buffered saline
pg	picogram
Ph	degree of alkalinity or acidity
PMSG	pregnant mare's serum gonadotropin
pro	proestrus
R	red
RIA	radioimmunoassay
RNA	ribonucleic acid
s.a.	sialic acid
sc	subcutaneous
T	thionin
TC	total counts
tri	three
w/v	weight/volume
x	times
XS	cross section
ZIFT	zygote intrafallopian transfer
±	plus or minus
≤	less than or equal to
°C	degrees Celsius
µL	microlitre

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Figure 1. Quotation

Johann Wolfgang von Goethe (1749-1832)

Wir wissen nur, wie wenig wir wirklich wissen,

mit Wissen kommt Zweifel.

1.0 INTRODUCTION

1.1 Female infertility and its treatment

Infertility affects 6-18% of the human population (Winston, 1991). The birth of Louise Brown in 1978 demonstrated the success of human in vitro fertilization (IVF) (Steptoe & Edwards, 1979). Now IVF and embryo replacement (ER) have become established as a treatment for some causes of female infertility (Wood & Trounson, 1989) and may also aid in the replenishment of endangered mammalian species (Bavister, 1982). Prevalent reproductive techniques that also aid in the treatment of infertility include: Artificial insemination (AI), collected semen is placed directly into the uterus (Bromham & Lilford, 1991); gamete intrafallopian transfer (GIFT) the egg and sperm are placed in the fallopian tube where fertilization is facilitated, zygote intrafallopian transfer (ZIFT), the zygote is placed in the fallopian tube where zygote transport is facilitated (Winston, 1991); and ER or embryo transfer (ET), the embryo is placed in a suitably primed uterus. ER in humans is used in conjunction with IVF and the embryos are usually transferred into the uterus of the women from whom the oocytes were obtained.

Nonprimate female mammals are often superovulated and then artificially inseminated. Preimplantation embryos are then retrieved from the uterus and placed in the uteri of other synchronized recipients. Commercially, ET has been used for rapidly multiplying the number of offspring from desirable dairy cattle, horses, sheep and pigs (Betteridge, 1977). In particular, ET has been used in the cattle industry to: multiply exotic cattle, obtain progeny from genetically superior or infertile cows and prepubertal heifers, build herds from a few superior cows, provide embryos so that commercial beef cows can be twinned and transport embryos instead of adult animals (Elsden, 1977).

Superovulation with exogenous gonadotropins is commonly used to obtain increased

numbers of oocytes for IVF and ET programs (Leveille & Armstrong, 1989) and other reproductive research projects. Exogenous gonadotropins induce superovulation primarily through the recruitment of primordial follicles or by the rescue of early atretic follicles (Moon, Yun & King, 1990). Excessive estrogenic stimulation of the genital tract due to the hyperstimulation of the ovary may lead to an asynchrony between the blastocyst and the development of the endometrium (Miller & Armstrong, 1981; Walton, Huntley, Kennedy & Armstrong, 1982). The luteal phase of IVF-ET/GIFT cycles has been studied as a potential cause of implantation failure because the rate of successful fertilization of human oocytes is approximately 80-90%, but the pregnancy rate is only 20% (Oehninger & Hodgen, 1990).

1.2 The Uterus

The uterus is a dynamic organ normally regulated by a procession of cellular and molecular events in response to the cyclic secretion of ovarian estradiol and progesterone (Leavitt, 1989). The functions of the uterus include:

a. facilitating the transport of sperm into the oviducts,

b. producing fluids that provide a favourable environment for sperm capacitation and for sustaining the preimplantation blastocyst,

c. participating in the formation of the placenta after implantation,

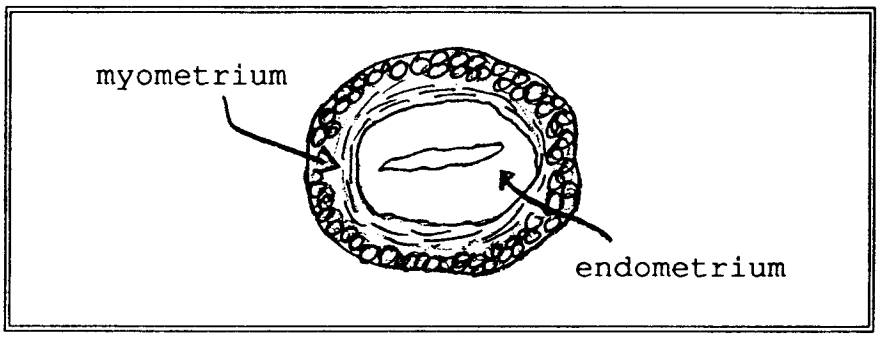
d. providing the site of fetal development, and

e. playing a major role in parturition (Hafez, 1970).

Physiologically, 2 layers of the uterus are recognized (Figure 2a): the myometrium and the endometrium (Hafez, 1970). The myometrium consists of a thick inner circular smooth muscle layer and a thinner outer smooth muscle layer composed of muscle cells embedded in connective tissue. This arrangement facilitates contractile transmission generated by individual muscle cells. During pregnancy, the myometrium relaxes to accommodate the

developing fetus, but near term, the myometrium provides the rhythmic tonic contractions of labour that expel the uterine contents (Huszar & Walsh, 1989). The endometrium is comprised of the surface epithelial lining of the uterine lumen and glands as well as the subepithelial stromal layer with supportive connective tissue. The thickness and vascularity of the endometrium are predominantly influenced by the ovarian steroid hormones, estradiol and progesterone.

Figure 2a. Schematic diagram of the rat uterus in cross-section



1.2.1 The structure of the rat uterus

Anatomically, the rat uterus is classified as a duplex uterus (Hebel & Stromberg, 1986). The lumina of the uterine horns are completely separate and open as a pair into a single vagina. The horns are partially fused caudally and share a common outer longitudinal myometrial layer. The uterus is a heterogeneous organ with several characteristic cell types; luminal and glandular epithelial cells, stromal cells, fibroblasts, two or more layers of smooth muscle, and vascular elements. The mesometrial triangle refers to the area enclosed by the inner circular muscle and the outer muscle layer where the large blood vessels occur (Lobel,

Liliane & Shelesnyak, 1965). The uterine lumen runs from the mesometrial region to the anti-mesometrial region.

The luminal surface is lined with simple columnar epithelial cells that form low longitudinal folds and wide crypts. The epithelial cells have distinct apical and basolateral domains which have very different functions. The apical epithelial surface supports embryo attachment and mediates secretory activity into the uterine lumen. In contrast, the basal surface must maintain contact with the basement membrane and mediate interaction with the subepithelial stroma (Carson, 1989). The glands are simple tubular and near the middle of the fused portion of the uterus, (the uterine cervix) there is a transition to a nonglandular stratified squamous epithelium. This area undergoes the same cyclic changes as the vaginal epithelium during the estrous cycle (Hebel & Stromberg, 1986).

1.2.2 The rat estrous cycle

The word "estrus" is a Latin adaptation of the Greek word "oistros," meaning gadfly, sting or frenzy. This term was first used by Heape in 1900 to describe the "special period of sexual desire of the female." Heape used the prefixes pro-, di-, and met- with the suffix estrus, to describe the stages of the cycle between the periods of estrus. Proestrus refers to when an animal is "coming on heat," while the next period, estrus, was described as the period of time that the female rat was willing to receive the male. Estrus is then followed by metestrus, during which the estrus changes in the reproductive tract subside. During diestrus, the ovarian secretions prepare the reproductive tract for the receipt of the blastocyst after mating during estrus. If there is no conception, the cycle repeats itself (Freeman, 1988). In the estrous cycle, the proliferated uterine mucosa is absorbed. This is in contrast to the human menstrual cycle, where the proliferated uterine mucosa is sloughed from the uterine wall.

Laboratory rats are nonseasonally polyestrous and ovulation occurs spontaneously every 4-5 days. The lengths of the various stages of the estrous cycle based on vaginal cytology are: proestrus (12-14 h), estrus (25-27 h), metestrus (6-8 h) and diestrus (55-57 h) (Freeman, 1988).

1.2.3 Steroid hormone action on rat endometrium

The uterine response to ovarian hormones may be classified into three categories, each controlled by a different mechanism. In the first category, hormones (especially estrogen) induce genomic changes such as increased RNA, and increased specific enzymes and protein synthesis accompanied by biochemical, morphological and functional differentiation of the uterine cells. The second category of uterine response involves nongenomic changes and includes hormone induced uterine eosinophilia, edema, vascular permeability, histamine release and uterine luminal fluid accumulation. The third category of hormone-induced uterine changes includes hyperemia, increased glycogen content and mitotic response (Leavitt, 1989).

Estradiol is responsible for the increase in uterine DNA (Anderson & Musah, 1989), DNA-dependent RNA synthesis, glycogen and phospholipid metabolism, synthesis of alkaline and acid phosphatase and mitotic activity (Leavitt, 1989). During proestrus, ovarian estradiol secretion is reflected in the uterus by the proliferation of the epithelial, stromal and myometrial cells. After ovulation, progesterone is responsible for increasing subepithelial stromal cell replication and for stimulating the synthesis and release of mucopolysaccharides, lipids, glycogen and hydrolytic enzymes (Wynn, 1989). Progesterone also maintains a block on myometrial excitability (Hamoir, 1977).

1.3 Pregnant Mare's Serum Gonadotropin (PMSG)

1.3.1 Characteristics

Several decades ago, it was discovered that mare's serum from various stages of pregnancy stimulated ovarian growth in immature rats and mice (Cole, 1936). The active ingredient in the pregnant mare's serum is a glycoprotein hormone, equine chorionic gonadotropin, often called PMSG (Armstrong & Leung, 1990). PMSG has both follicle stimulating hormone (FSH) and luteinizing hormone (LH) biological activity in species other than the horse (Murphy & Martinuk, 1991). Therefore, it is a potent agent for the induction of follicular development in both domestic and laboratory mammals.

PMSG is a heterodimeric glycoprotein hormone that has dissimilar α and β subunits that are noncovalently complexed. The α subunit is common to LH, FSH and thyroid stimulating hormone (TSH). The variation in the β subunit between the hormones imparts the different biological activities. PMSG is the most heavily glycosylated mammalian pituitary or placental glycoprotein with approximately 45% of its mass attributable to carbohydrate moieties. The circulating half-life of PMSG is 36 hours (Murphy & Martinuk, 1991). In contrast, the circulating half-life of FSH and LH is only 2.5 hours and 0.5 hours, respectively (Moon et al., 1990). The extended half life of PMSG is due to its higher sialic acid content.

1.3.2 Physiological effects of PMSG on ovulation in rats

PMSG effects the ovary both indirectly and directly (Yun, Ho Yuen & Moon, 1987). A single injection of PMSG administered to female rats from approximately day 20 of life induces an increased gonadotropin and steroid secretion that leads to ovulation (Parker & Mahesh, 1976). A physiological dose of PMSG stimulates ovulation indirectly through the endogenous LH surge that is initiated due to follicular maturation and steroidogenesis

(Wilson, Horth, Endersby & McDonald, 1974; Parker, Costoff, Muldoon & Mahesh, 1976). Physiological doses of PMSG also have a direct effect on the ovary by initiating the maturation of the preovulatory follicles with the largest number of FSH receptors (Moon et al., 1990).

When the hypothalamic-pituitary-ovarian axis is intact, there is an increase in LH, estrogen and progesterone concentrations in the periovulatory period. The serum estrogen concentrations in immature rats increase for 20 - 24 hours and then level off approximately 48 hours following the administration of a physiological PMSG dose (Wilson et al., 1974). Then, there is an increase in hypothalamic gonadotropin releasing hormone concentrations and a stimulation of FSH, LH and prolactin release from the pituitary (Sashida & Johnson, 1975). The secretion of various ovarian and adrenal steroids, including progesterone and testosterone, occurs 52-56 hours after PMSG injection (Wilson et al., 1974; Parker et al. 1976). A "physiologic" dose of 4 - 8 IU PMSG will induce synchronous ovulations in immature rats, beginning 60 h post administration. The yield of 8-12 oocytes over a 12 hour period is similar to that found in mature rats (Wilson et al., 1974; Walton & Armstrong, 1983; Yun et al., 1987; Moon et al., 1990).

In contrast, a "superovulatory" dose of 20 or 40 IU PMSG has a different mechanism of action on the ovary. Superovulatory doses of PMSG induces ovulation over a period ranging from 24 to 72 hours after administration (Walton et al., 1983; Yun et al., 1987). There are two distinct groups of oocytes released. The first group of oocytes are released 12-36 hours following a 20-40 IU dose of PMSG. An elevated concentration of estradiol is not associated with this first set of ovulations and without this elevated estradiol suggests that PMSG initially acts directly on the ovary (Fang, 1988; Moon et al., 1990). The second set of ovulations 48 hours post injection may be due to an indirect effect of PMSG. The basal estrogen and progesterone secretion from the follicles from the first set of ovulations may now

synergistically stimulate the LH surge (Ying & Meyer, 1969). Therefore, the ovulations that occur within 48 hours of superovulatory doses of PMSG injection may be independent of the pituitary gland, but the subsequent ovulations are dependent on the endogenous LH surge (Moon et al., 1990).

1.3.3 Implantation in rats

Implantation refers to the attachment of the embryo to the uterine wall and the establishment of a functional union between maternal and fetal tissues to provide nutrients to and remove waste products from the fetus (Parr & Parr, 1989). In preparation for blastocyst implantation, each endometrial cell type performs a different function. The luminal epithelium provides the initial surface to which the trophoblast cells attach; the glands provide secretions that nourish the blastocyst before placentation; the stromal cells differentiate into specialized decidual cells. The term uterine sensitivity relates to the capacity of the endometrium to produce the implantation reaction. The endometrium is receptive to the blastocyst for only a very short period and then becomes inhospitable. The changes that render the endometrium refractory may occur at several levels and may include changes in the luminal secretions or the loss of apical attachment sites. It is also important that the blastocyst be at the correct stage of development, otherwise implantation will not occur (Finn, 1977).

Uterine sensitization begins a few days following ovulation, and both estradiol and progesterone from the ovary as well as growth factors such as insulin-like growth factors (IGF) and epidermal growth factors (EGF) are essential for the development of endometrial sensitivity. The rat endometrium must be exposed to progesterone for a minimum of 48 hours in order to develop a uterus that can accommodate the presence of embryos. Then when a single minimal dose of estradiol is administered, the rat uterus will be receptive for

blastocyst attachment for a period lasting 12-36 hours. After the rodent uterus is "sensitized," the transformation of stromal cells into decidual cells may occur in response to blastocyst implantation or to various artificial stimuli (ie. trauma, scratching or crushing of the endometrium) (Finn, 1977).

Implantation may be considered as a sequence of biochemical and physical interactions between the embryo and the uterus which can be separated into three phases: apposition, adhesion and penetration (Lindenberg, 1991). The blastocysts are spaced along the length of the uterus to prevent the blastocyst from implanting too close together. This spacing may be the result of myometrial activity, the local production of prostaglandins, estradiol or the differential growth of the uterus (Finn, 1977). The breakdown and removal of the zona pellucida from the blastocyst is brought about by the secretion of enzymes from the uterus. Successful apposition and adhesion is believed to require a reduction of the negative charge on both the uterine and the blastocyst surfaces by the removal or masking of terminal sialic acid residues on the surface carbohydrate (Parr et al., 1989). However, there is no detailed biochemical mechanism for blastocyst adhesion known.

Active endocytosis in the surface uterine epithelial cells during the preimplantation period may remove selected glycoproteins from the apical membrane. There is a large population of apical vesicles present during pre-implantation and these may fuse with the apical membrane and deliver newly synthesized components needed for adhesion (Parr et al., 1989). Attachment is defined as the stage at which a functional close relationship is formed between the outer membrane of the trophoblast cells and the luminal epithelium. Any change on the surface of the cells leading to attachment is probably associated with the complex carbohydrate moieties of the glycoproteins, glycolipids and glycosaminoglycans located at the cell surface in the glycocalyx (Lindenberg, 1991).

In the rat, the type of implantation is classified as displacement penetration (Parr et

al., 1989). This type of implantation involves the trophoblast processes extending between and beneath the surface epithelial cells. The epithelial cells or fragments are phagocytized and the trophoblast then lays on the basal lamina which was previously occupied by the displaced uterine cells. The first observed response of the endometrium to implantation is an increased capillary permeability followed by the decidualization of the stromal cells adjacent to blastocysts. Implantation occurs at or near the antimesometrial end, with the embryonic disc facing the mesometrium (Finn, 1977).

In the human, no information on the time and mode of attachment and initial penetration has been obtained. Attempts to extrapolate animal data to explain human implantation mechanisms have failed and it is clear that comparisons can only be made with caution, even between closely related species (Lindenberg, 1991). An alternative approach has been to investigate an in vitro implantation model. An intriguing feature of human in vitro implantation was the finding that the polar trophoblast displaced endometrial cells in the central part of the implantation site. It is not clear whether this was the result of pushing by the invading trophoblast or by a local redistribution of the endometrial cells themselves, as is seen in the rat (Lindenberg, 1991).

Blastocysts are incapable of implantation into a uterus which has not been properly sensitized by ovarian steroid hormones. However, they are capable of implantation at extrauterine sites regardless of the hormonal environment. It is possible that the apical surface of the non-receptive uterine luminal epithelium expresses molecules which prevent the blastocyst from invading the uterus. The steroid hormones induce specific molecular changes in the luminal surface of the uterus to allow the trophoblast to adhere to and then penetrate and invade the endometrium (Svalander, Odin, Nilsson & Obrink, 1990).

1.3.4 Significance of carbohydrates in uterine physiology

The secretions from the endometrium are under hormonal control and provide an optimal environment for the transport and capacitation of spermatozoa and nutrition for the preimplantation blastocyst (Hafez & Ludwig, 1977). The endometrial fluid is derived from serum transudate proteins, carbohydrates and other metabolites synthesized within the endometrial cells and discharged through apical cell membranes. Previous studies have shown that the carbohydrate complexes of the luminal uterine surface change during the time of attachment and the adhesive phases (Lindenberg, 1991).

1.3.4.1 Neutral sugars

Recent studies suggest that there are specific oligosaccharide determinants on the surface of the uterine epithelial cells which appear around the time of implantation due to hormonal induction (Lindenberg, 1991). The surface charge on the blastocyst appears to be associated with glycoproteins and N-acetylneuraminic acid (sialic acid) which are present in the terminal position of the cell surface carbohydrates. The decreased negativity of the blastocyst surface at the time of implantation is apparently independent of maternal influences (Parr et al., 1989).

The immature rat uterus does not respond to estrogen in the same manner as the mature rat uterus (Carson et al., 1989). N-linked glycoprotein assembly is stimulated by estrogen in both immature and mature mice. In mature mice, a subset of a N-linked oligosaccharide, the lactosaminoglycans (LAGs) are major cell surface components of uterine epithelial cells. However, uterine epithelial cells in immature mice synthesize LAGs which are not N-linked. Additional maturational influences are required to endow these cells with the ability to synthesize this class of glycoproteins.

1.3.4.2 Sialic acids

Sialic acids are an essential constituent of many glycoproteins, glycopeptides and glycolipids. This widespread occurrence in glycoconjugates of different origins indicates that this sugar may have a variety of biological functions. Sialic acids may influence or determine the recognition of low and high molecular-weight compounds, the action of certain hormones, physicochemical and catalytic properties of enzymes, haemostasis, cellular adhesiveness, antigenicity, transport processes and synaptic transmission. Sialic acid has four main functions:

a. responsible for the negative charge of many cells and soluble glycoconjugates, thus preventing aggregation, agglutination or precipitation,

b. influences the macromolecular structure of certain glycoproteins,

c. provides recognition sites for information transfer, and

d. protects glycoconjugates and cells from recognition and premature degradation (Schauer, 1982).

The surface negative charge on uterine epithelial cells at the time of implantation has been characterized by the use of electron microscopy in combination with cytochemical markers such as ruthenium red. The intensity of such labelling was completely lost in rats during implantation, but attempts to assess the changes in negativity are ambiguous. It is possible that the reduced negativity may be caused by a decreased amount of sialic acid residues. However, the role of the changes in surface charge or surface coat in blastocyst adhesion to the uterine epithelium remains speculative.

The content of sialic acid on the plasma membrane may be subject to hormonal regulation. It is conceivable that changes in hormonal concentrations could influence cellular interaction by altering the amount of membrane-bound sialic acid (Schauer, 1982).

1.3.4.3 Sulphate esters

The endometrium synthesizes and secretes sulphate glycoconjugates which may play an important role in the transport and implantation of the blastocyst (Mahfoudi, Beck, Nicollier, Coosemans & Adessi, 1991). The secretion of sulphated uterine glycoproteins is increased by steroid hormones, especially estradiol (Takata & Terayama, 1977). Progesterone in concert with estradiol, increases the available uterine intracellular sulphate pool. It has also been suggested that a sulphate transport system may be involved in the accumulation of intracellular sulphate. This may be an early process in the preparation of the endometrium for implantation. Sulphate residues may also compensate for the absence of sialic acid in formation of a protective layer of negative charges.

Little is known about the regulation of glycoprotein expression by the uterine stromal cells. A number of studies have shown that stromal cells begin to synthesize different glycoconjugates associated with cell adhesion processes as part of the decidual response (Carson et al., 1989). The changing pattern of stromal glycoprotein expression may have important influences on the ability of the embryo to invade the endometrium. The regulation of the expression of glycoproteins by the embryo appears to be under developmental control. Therefore, in the uterus, glycoprotein expression changes in response to a number of factors and includes effects on the glycosylation apparatus as well as effects on glycoprotein mRNA expression. These uterine processes are influenced strongly by the hormonal milieu, the sexual maturity of the animal, the state of cell polarity in the case of epithelial cells as well as by uterine cell-cell interactions, that are poorly understood (Carson et al., 1989).

1.4 The immature rat model

1.4.1 Ovulation and pregnancy

The immature rat model has been used for reproductive studies since 1962 when Straus and Meyer used 8 IU of PMSG to induce synchronous ovulation in prepubertal rats. The hormonal changes associated with ovulation in this model (serum estradiol, progesterone, LH) are similar to mature rats (Meijs-Roelofs, Uilenbroek, De Greef, DeJong & Kramer, 1975; Parker et al., 1976). Furthermore, in immature rats, pseudopregnancy, decidualization, maintenance of pregnancy and post-partum reproductive performance were comparable to that found in mature adult rats (Nuti, Sridharan & Meyer, 1975).

1.4.2 Advantages and disadvantages of the immature rat model

A number of studies provide evidence that immature PMSG-treated rats are suitable substitutes for adults rats in studies of reproduction, particularly those requiring large numbers of synchronized animals (Nuti et al., 1975). The immature rat model is preferable to the adult rat reproductive cycle due to the potential variability in the length of the estrous cycle and the difficulty of synchronizing ovulation in adult rats. Furthermore, the immature rat model is relatively convenient, inexpensive and may be less time consuming than using an adult model (Nuti et al., 1975). Another advantage of using the rat model is that the process of implantation in rats may be similar to the implantation process which occurs in humans (Lindenberg, 1991).

Disadvantages of using the laboratory rat as a model include differences in the physiology and anatomy among humans or domestic mammals (ie. cow, horse, pig); the type of reproductive cycle (estrous versus menstrual); the length of reproductive cycle (ie. 4 d in rat versus 28 d in humans); the shape of the uterus; and the functions of the uterine cellular components (ie. stromal cells).

1.5 Rationale

Superovulation with pregnant mare serum gonadotropin is associated with a decreased pregnancy rate in immature rats (Walton et al., 1982). Oocytes removed from superovulatory PMSG treated immature rats are capable of being fertilized and when replaced into a suitably primed uterus will develop into normal offspring. Thus, the failure of superovulatory treated immature rats to maintain a pregnancy may not be due to defects in the oocytes or embryos (Walton et al., 1982; Walton & Armstrong, 1983). In fact a study of rats treated with 40 IU PMSG, showed that there were no blastocysts in the uterus on Day 5 of pregnancy (Miller & Armstrong, 1981). This lack of blastocysts suggests that the uterine environment was not suitable for successful implantation to occur.

It has been shown that successful implantation and pregnancy may be disrupted if the uterine morphology or carbohydrate composition are altered (Finn, 1977). Therefore the purpose of this study was to examine the effects of a 4 IU PMSG (physiological) dose to a 40 IU PMSG (superovulatory) dose on the uterine environment by examining the morphology of the uterine epithelium, the carbohydrate composition of the glycocalyx on the apical surface of the epithelium and the uterine secretions in 4IU PMSG treated and 40 IU PMSG treated immature rats.

1.5.1 Hypothesis

The objective of this study was to test the hypothesis that abnormalities in the morphology of the uterine epithelium, changes in the amount and composition of the carbohydrate components of the uterine glycocalyx and secretions are responsible for the decrease in fertility seen in 40 IU PMSG superovulated immature rats.

1.5.2 The aims of the project

The specific aims of this project was to answer the following questions:

1. Does the uterine morphology in 40 IU PMSG (superovulatory dose) treated immature rats differ from that in 4 IU PMSG (physiological dose) treated immature rats or untreated mature rats?
2. Does the carbohydrate composition of the glycocalyx and uterine secretions in 40 IU PMSG (superovulatory dose) treated immature rats differ from that in 4 IU PMSG (physiological dose) treated immature rats or untreated mature rats?
3. Does the amount of the glycocalyx and uterine secretions in 40 IU PMSG (superovulatory dose) treated immature rats differ from that in 4 IU PMSG (physiological dose) treated immature rats or untreated mature rats?
4. Does the serum steroid response (estradiol, progesterone, testosterone) in 40 IU PMSG (superovulatory dose) treated immature rats differ from that in 4 IU PMSG (physiological dose) treated immature rats or untreated mature rats?

2.0 MATERIALS AND METHODS

The chemicals and instrumentation used in this study are divided by technique and are listed by supplier.

Experimental animals were injected with Pregnant mare serum gonadotropin (PMSG, Equinex, Ayerst, Montreal, QUE).

2.1 Radioimmunoassay (RIA)

The chemicals used for RIA included the following: sodium chloride, sodium bicarbonate, crystalline monobasic sodium phosphate, anhydrous dibasic sodium phosphate, sodium azide, purified crystalline progesterone, estradiol and testosterone (Sigma, St. Louis, MO); tritiated tracers [1,2,6,7,16,17-^{3}H] progesterone, [2,4,6,7,16,17-^{3}H] estradiol and [2,6,7-^{3}H] testosterone (Amersham, Oakville, ONT); anhydrous diethyl ether (BDH Limited, Toronto, ONT); carbon decolorizing alkaline norit A, granular gelatin bloom, Scintiverse E Universal LSC cocktail, scintillation counter LKB 1217 RackBeta (Fisher Scientific Company, Fair Lawn, NJ); ethyl alcohol (Stanchem Inc., Vancouver, BC); radioassay (Tri-Rac-R-Dade) controls (Baxter Diagnostics Corporation, Canlab Division, Toronto, ONT) and miniature polyethylene vials (Canberra-Packard, Groningen, Netherlands).

The specific antisera for estradiol, progesterone and testosterone were supplied by Dr. David T. Armstrong (University of Western Ontario, London, ONT). The cross-reactivity of the steroid antiserum used are shown in Table 1.

Table 1. Cross-reactivity of steroid antisera

progesterone	100%
5β-pregnane-3,20-dione	35.5 %
5α-pregnane-3,20-dione	15.7 %
3α-hydroxy-5β-pregnan-20-one	2.0 %
20β-hydroxy-4-pregnen-3-one	1.3%
17α-hydroxyprogesterone	1.2%
other major steroids	less than 0.2%
estradiol	100 %
estrone	2.9 %
estriol	0.5 %
other major steroids	less than 0.2%
testosterone	100 %
5α-dihydrotestosterone	75 %
5α-androstane-3α,17β-diol	13.5 %
5 -androstane-3β,17β-diol	10.9 %
19-hydroxytestosterone	4.7 %
other major steroids	less than 1 %

2.2 Chemicals for histological and histochemical procedures

2.2.1 Histology

The chemicals used for the histological methods included: formaldehyde 37-41% solution, 3-aminopropyl triethoxysilane, glacial acetic acid, aluminum sulphate, lithium carbonate (BDH Inc., Toronto, ONT); hydrochloric acid, xylene (histological grade), permount, picric acid, (Fisher Scientific, Fair Lawn, NJ); paraplast tissue embedding medium (Baxter Diagnostics Corporation, Canlab Division, Toronto, ONT).

2.2.2 Histochemistry

The chemicals used for the histochemical methods included: eosin (C.I. 45380), periodic acid 99%, pararosaniline hydrochloride (C.I. 42500), thionyl chloride, alcian blue 8GX (C.I. 74240), nuclear fast red, sodium borohydride (BDH Inc., Toronto, ONT); potassium hydroxide, Gills II hematoxylin, sodium iodate, ethylene glycol, and certified alum sulphate crystal (Fisher Scientific, Fair Lawn, NJ).

2.2.3 Histological instrumentation

The equipment and instruments used for histology included: disposable Feather S35 microtome blades, tissue prep flotation bath, slide warmer (Fisher Scientific, Fair Lawn, NJ); superfrost microscope slides, microscope coverslips, (Johns Scientific, Toronto, ONT); Tissue Tek II and III process/embedding cassettes, mold release, Tissue-Tek cryoconsole and dispensing console (Ames Division, Miles Laboratory, Naperville, IN); Shandon Citadel 2000 Tissue processor (Shandon Inc., Pittsburg, PA); Reichert-Jung 2030 Biocut rotary microtome (Nubloch, Germany); Bioquant II digital morphometry computer program (A & M Biometrics); Nikon labophot biological microscope, (Nikon, Japan).

2.3 Experimental animals

Immature (21 day) and mature (2 months) female Sprague-Dawley rats (Charles River Canada, St. Constant, QUE) were kept under controlled conditions of temperature (21-25°C) and light (12 h light: 12 h dark cycle; lights on at 07:00 h) and provided with free access to standard rat chow and water.

2.4 Methods

2.4.1 Experimental design

Experiments were initiated when immature rats reached 28 d (Table 2). Immature female rats (total n = 114) were randomly divided into 2 groups and were subcutaneously (sc) injected with a single dose of either 4 IU PMSG in 0.4 ml of 0.9% saline (physiological group n = 54) or 40 IU PMSG in 0.9% saline (superovulatory group n = 54). The rats were weighed and sacrificed by decapitation at time 0, 24, 48, 72, 96, 120, 144, 168, 192 and 216 h post PMSG injection (n = 6 /group). Mature rats were sacrificed when in the desired stage of the estrous cycle. Trunk blood was collected into glass tubes and stored at 4°C for 4 - 12 hours

prior to separation by centrifugation. The serum was kept frozen at - 20°C until extraction and RIA for estradiol, progesterone, and testosterone.

Table 2. Experimental design

Hours after PMSG injection	Number of immature rats treated with 4 IU PMSG (physiological dose)	Number of immature rats treated with 40 IU PMSG (superovulatory dose)
0	6 (no injection)	-
24	6	6
48	6	6
72	6	6
96	6	6
120	6	6
144	6	6
168	6	6
192	6	6
216	6	6
Total	54 (+ 6 with no injection)	54

Stage of cycle	Proestrus	Estrus	Metestrus	Diestrus
Number of mature rats	3	4	3	3

2.4.2 Preparation of tissue for histology and histochemistry

The rat abdomen was surgically opened mid sagitally. The ovaries and uterus with vagina were dissected free from each other and cleaned of bursae, connective tissue and fat. They were kept in petri dishes on a cryotable (-6 to -2°C), and then were blotted and weighed. The uteri were attached to a paraplast block with stainless steel pins and fixed in Bouin's fixative (75 mL saturated picric acid, 25 mL 40% formalin, 5 mL glacial acetic acid) for 2 h.

The uteri were then washed with tap water and stored in 70% ethanol. One uterine horn was cut into three 0.5 cm long pieces (0.5 cm from oviduct end; midway down the uterus and at the utero-cervical external junction) (Figure 2b). The three segments were marked with a drop of either India ink (middle segment) or mercuric chrome (cervical end segment) to ensure correct identification and orientation of the tissue. The uterine-cervix and vagina segment were cut in half longitudinally. The 4 pieces of tissue were placed in tissue tek cassettes. The tissues were dehydrated through graded alcohols, (70%, 6 h; 85%, 1 h; 2 x 95%, 1 h; 3 x 100% 1 h;) cleared in xylene (3 x 45 min) and infiltrated with paraplast (2 x 1 h). The three segments of the uterine horn were embedded transversely in a systematic pattern around the longitudinally oriented uterine-cervix-vagina (Figure 2c). Serial sections of the uterine horn in the cervical, middle and oviductal regions, the uterine-cervix and vagina region were cut at a thickness of 4 microns. Control sections of liver and intestine from both mature and immature rats, as well as rat salivary gland were also obtained. Sections were floated out onto a thermostatically controlled waterbath at 42°C and picked up on silanized treated (2% 3-aminopropyl triethoxysilane in 100 mL acetone for 10 min and rinsed in tap water) microscope glass slides. The sections were placed in a 60 °C oven for 1 hour.

Figure 2b. Schematic diagram of the rat uterus

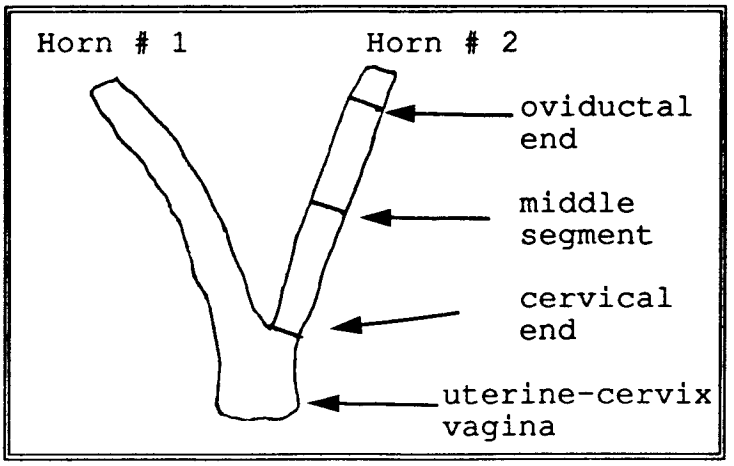
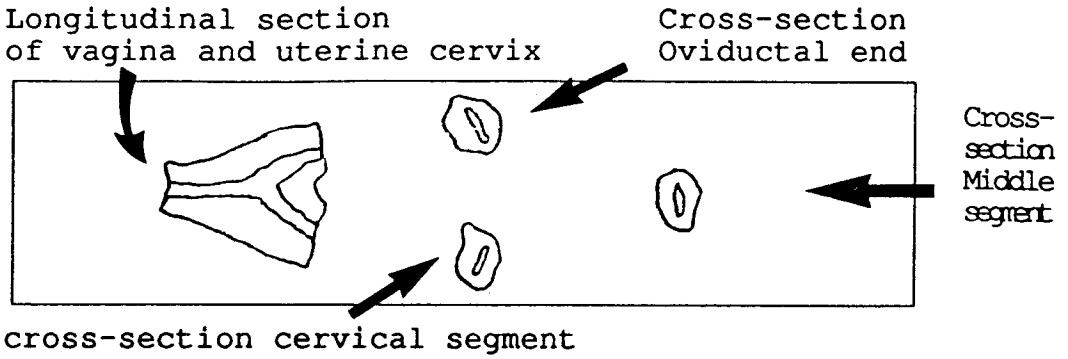


Figure 2c. Schematic diagram of the orientation of uterine segments on slide



2.4.3 Histochemical analysis

Histochemical procedures for the specific demonstration of carbohydrates, (neutral sugars, glycogen, O-sulphate esters, and sialic acids with and without O-acyl side chain substituents) were performed on the uterine horn cross sections (XS) and longitudinal uterine-cervical-vaginal sections.

In all the methods, the test sections and the appropriate controls were brought to water, dipped ten times in 2% aqueous sodium bicarbonate to remove picric acid and then rinsed well with water. The sections were then distributed randomly into 5 large glass staining dishes (20 per dish). All the sections were treated in the same way; exposed to the same freshly prepared reagents for the same length of time. After completion of staining, the sections were washed in running tap water for 10 min, dehydrated through graded alcohols, cleared in xylene and mounted in permount.

2.4.4 Histochemical procedures

2.4.4.1 Pararosaniline Schiff (S) and Thionin Schiff (T) (Barger and De Lamater, 1948) were prepared immediately before use. Staining was performed for one hour at r.t. and the sections were then drained.

2.4.4.2 Alcian blue 8GX pH 1.0 (AB1.0) (Culling, 1974) were prepared as a stock solution and filtered before each use. Staining was performed for 30 minutes at r.t. The sections were drained thoroughly, rinsed twice with a solution of 0.1 N HCl at pH 1.0, and then drained again.

2.4.4.3 Periodate oxidation was performed with either:

a) 1.0% (w/v) aqueous periodic acid (PA) for 2 hours at r.t. (Culling & Reid, 1977) or

b) a pre-cooled (4°C) solution of 0.4 mM periodic acid (PA*) in 1 M hydrochloric acid for 1 hour at 4°C (Volz et al., 1987).

2.4.4.4 Borohydride reduction (Bh) was performed by using a fresh 0.1% sodium borohydride in 1% disodium hydrogen phosphate solution for 30 minutes at r.t. (Lillie & Pizzaloto, 1972).

2.4.4.5 Saponification (KOH) was performed by initially rinsing the sections a solution of 70% ethanol and then using a freshly prepared solution of 0.5% (w/v) potassium hydroxide in 70% ethanol for 15 min at r.t.

2.4.4.6 Salivary amylase was diluted (1:3) with distilled water and the sections immersed for 1 h at 37°C and then rinsed in running tap water for 10 minutes.

2.4.5 Staining methods

The following staining methods were used (see Table 3):

1. The PAS for the detection of periodate oxidizable vicinal diols was performed with 1% periodic acid.
2. The PA*S for the selective detection of sialic acids with periodate oxidizable vicinal diols was performed with a solution of 0.4 mM periodic acid (Volz et al.,1987a,b).
3. The AB1.0 for the detection of 0-sulphate esters was performed with Alcian Blue at pH 1.0 and sections counterstained with 0.1% nuclear fast red in 5% w/v aluminum sulphate for 5 min at r.t. (Culling, 1974).
4. Neutral sugars were detected before and after digestion with salivary amylase using the PA*/Bh/PAS procedure (Reid et al., 1990). The enzyme amylase selectively removes glycogen. The reduction in the intensity of the red staining in comparison to the water/PA*/Bh/PAS treated section, indicates the presence of glycogen.
5. The PA*/T/Bh/PAS/KOH selectively stains sialic acids without carbon side chain substituents or which are substituted at position C7, blue. Neutral sugars with oxidizable vicinal diols stain red. Mixtures of the sialic acids and neutral sugars stain various shades of purple dependent upon their relative concentrations. This method

enabled a semiquantitative ratio of sialic acids to neutral sugars to be determined (Reid et al., 1987).

6. The PA*/Bh/AB1.0/PAS selectively stains neutral sugars a red colour and O-sulphate esters an aquamarine colour. Mixtures of these components stain various shades of purple which enabled a semiquantitative ratio of neutral sugars to O-sulphate esters to be determined (Volz et al., 1987a,b).

7. The PA*/AB1.0/S selectively stains sialic acids without side chain O-acyl substituents or sialic acids with an O-acyl substituent at position C7, red. This method also selectively stains O-sulphate esters an aquamarine colour. Mixtures of the sialic acids and sulphate groups stained various shades of purple depending upon the relative concentrations. This method allows a semiquantitative ratio of sialic acids to sulphate groups to be determined (Reid et al., 1987).

2.4.6 Control sections

Staining was controlled by the use of appropriate tissue sections for the method performed. Sections of formalin fixed and Bouin fixed liver and intestine were used in all methods. Rat salivary gland was used in the methods which detected sialic acid. Sections were also run with Pararosaniline schiff reagent alone to ensure that there were no pre-formed aldehydes. All the control sections stained as anticipated (Table 3) which indicated that the reagents and procedures performed as expected.

Table 3. Characteristics of the histochemical stains used for the identification of rat uterine glycoproteins

Method (Reference)	Sugars			Sulphate	Sialic acid		
	Neutral	Glycogen	0-acyl		C_0	C_7	C_8C_9
PAS	R	R	0	0	R	R	0
PA*S (Volz et al., 1987a,b)	0	0	0	0	R	R	0
PA*/Bh/PAS (Reid et al., 1990)	R	R	0	0	0	0	0
amylase/PA*/Bh/PAS	R	↓ R	0	0	0	0	0
PA/Bh/KOH/PAS (Reid et al., 1973)	0	0	R	0	0	R	R
AB1.0 (Culling, 1974)	0	0	0	A	0	0	0
PA*/AB1.0/S (Reid et al., 1987)	0	0	0	A	R	R	0
PA*/T/Bh/PAS/KOH (Reid et al., 1987)	R	R	0	0	B	B	0
PA*/Bh/AB1.0/PAS (Volz et al., 1987a,b)	R	R	0	A	0	0	0

Key: 0 = no staining R = red A = aquamarine B = dark blue
PA = Periodate oxidation; PA* = Selective Periodate oxidation
Bh = Borohydride reduction; KOH = Saponification; AB1.0 = Alcian Blue pH 1.0
S = Pararosaniline Schiff; T = Thionin Schiff
Neutral sugar = hexose, 6-deoxyhexose and N-acetyl hexosamine residues with periodate oxidizable vicinal diols
O-acyl sugars = neutral sugars that have an ester substituent on a potential vicinal diol (s)
sialic acid side chain: C_0 = no acyl side chain substituent;
C_7 = sialic acids with an O-acyl substituent at position C7;
C_8 & C_9 = sialic acids with an O-acyl substituent at C8 or C9
(or with two (C7C8, C7C9, C8C9) or three (C7C8C9) substituents;
amylase removes glycogen with a reduction in staining when sections are compared to the water/PA/Bh/PAS method

2.4.7 Methods of histochemical and histological assessment

Histochemical and morphological assessments were done on rat uteri from three rats from each group of 4 IU PMSG (n = 27), 40 IU PMSG (n = 27) treated immature rats and untreated mature rats in the stages of proestrus (n = 3), estrus (n = 4), metestrus (n = 3) and diestrus (n = 3). The immature rat uteri were selected randomly but only if they had a positive response to PMSG as determined by ovarian, uterine weight and steroid hormone response.

The histochemical methods used in this study permitted the semiquantative assessment of the relative amounts of sulphate esters, neutral sugars and sialic acid in the mature rat and PMSG stimulated immature rat. Although there was a vast amount of statistical tests done on a relatively small number of rats, a smaller significance value ($p<0.001$) was used to compensate for multiple comparisons. Also, the histochemical grading scale used was not exact.

2.4.7.1 Histochemical assessments

An assessment of the staining from the various histochemical methods were done on the following:

1. the free secretions in the uterine lumen,
2. the uterine lumen epithelial glycocalyx (apical surface lining),
3. the glandular epithelial glycocalyx and glandular contents,
4. the free secretions in the uterine-cervical-vaginal region,
5. the glycocalyx in the cervical-vaginal junction, and
6. the subepithelial stroma.

The "amount" of apical lining or secretion referred to the relative area which was

stained in all the staining methods used. The "amount" was graded on a scale of 0 (not present), 1 (trace), 2 (some), 3 (moderate) and 4 (maximal). The "colour" or "intensity" of the composition of the glycocalyx or secretions were graded according to the components stained by the single carbohydrate staining methods were graded in the same manner as "amount". In the PA*/T/Bh/PAS/KOH method, the grading used was: np (not present), 1 (blue = sialic acid), 2 (blue-purple), 3 (purple-blue), 4 (purple), 5 (purple-red), 6 (red-purple) and 7 (red = neutral sugars). In the PA*/AB1.0/S method, the grading used was: np (not present), 1 (aquamarine = sulphate esters), 2 (blue-aquamarine), 3 (blue), 4 (blue-purple), 5 (purple-blue), 6 (purple), 7 (purple-red), 8 (red purple) and 9 (red = sialic acid). In the PA*/Bh/AB1.0/PAS method, the grading used was: 1 (aquamarine = sulphate esters), 2 (blue), 3 (blue-purple), 4 (purple-blue), 5 (purple), 6 (purple-red), 7 (red-purple) and 8 (red = neutral sugars).

The colour or intensity interpretation of the secretions or glycocalyx were compared to other components of the uteri. (For example, in the methods containing Alcian blue, mast cells were present and were classified as a true aquamarine colour. Also, the red colour from the schiff reagent was usually present in the outer longitudinal muscle layers of the myometrium). This assisted in making a visual colour scale. The histological stains were assessed without prior knowledge of the time or group. However, in the uteri from the superovulatory group (after 96 hours), the endometrial morphology and amount of secretions were notably different, and therefore these results for the histochemical staining could not be assessed "blindly". Women see more shades of colour then men (Reid & Park, 1990), and it is likely that if observations of the uteri were repeated by myself, or by someone of similar training, the gradings would be similar, but not exactly the same.

2.4.7.2 Morphological assessments

Sections for morphological assessment were stained with hematoxylin and eosin. Particular attention was given to the endometrium, surface epithelial cells of the uterine lumen, the glands and the subepithelial stroma.

2.4.7.2.1 Measurement of luminal perimeters

The inner uterine luminal perimeter as well as the outer uterine perimeter was measured using digital morphometry. The image of the middle cross-sectional segment of the uterine horn was projected onto a screen using a microscope with a video camera attached. The surface of the inner uterine luminal epithelial cells as well as the outer uterine perimeter was traced and the lengths calculated using the Bioquant morphometric computer program.

2.4.7.2.2 Number of mitotic figures

The number of mitotic figures present in the uterine and glandular surface luminal epithelium or in the subepithelial stroma was determined by counting all the mitotic figures present in a 4 micron thick, middle uterine horn cross-section. The mitotic figures which were included in the counts displayed obvious chromosome patterns characteristic of late prophase to late anaphase (Wheater & Burkitt, 1987). The mitotic figures were counted in cooperation with Dr. B. Gilks, University Hospital, UBC site, Vancouver, BC., using a co-observation bridge and the consensus result recorded.

2.4.7.3 Determination of stage of the rat estrous cycle

The estrous cycle of 13 mature rats was assessed by vaginal exfoliative cytology over at least 3 cycles. A 1 ml syringe was used to perfuse the vagina with 1 ml of 0.9% NaCl. The saline containing exfoliated cells was placed on a glass slide and the cells assessed by light microscopy. Proestrus was characterized by the presence of individual or sheets of small nucleated epithelial cells, estrus was characterized by larger cornified epithelial cells, metestrus was characterized by increased leukocytes and reduced cornified epithelial cells and diestrus was characterized by the presence of mainly leukocytes (Hafez, 1970).

2.4.7.4 Determination of ovulatory response

The determination of the ovulatory response was accomplished by flushing the oviducts. The oviducts and uterine horns were separated at the uterotubal junction (only at times 0, 24, 48, and 72 h) and flushed with PBS by inserting a blunt 30 gauge needle through the infundibulum. The oocytes were counted under a dissecting microscope (40X) and examined by phase-contrast microscopy (100X).

Oocyte quality was morphologically assessed and was classified as either normal or abnormal. Normal oocytes were surrounded by cumulus cells, with the oocyte round with an even fine white colour appearance. Abnormal oocytes were those which displayed fragmentation or degeneration (yellow colour in middle of oocyte) (Lopata, Kohlman & Johnston, 1983). The oocyte flushing and assessment were done by Dr. Jun Young Hur and Dr. Sai Ma, Grace Hospital, Vancouver, BC.

2.4.7.5 Radioimmunoassays (RIA) of the steroid hormones

2.4.7.5.1 Preparation of the serum extracts

Aliquots of rat sera (0.4 - 0.7 mL) were diluted to a final volume of 1 mL with PBS (1.0 g gelatine, 9.0 g NaCl, 5.327 g NaH_2PO_4, 8.818 g Na_2HPO_4, 0.1 g NaN_3 in 1 litre, pH 6.9). The rat sera and undiluted radioassay controls Tri-Rac-R-Dade (1 mL) were extracted (by vigorous vortexing for 2 minutes) twice with 5 mL of diethyl ether. The extracts from each sample were combined, evaporated to dryness under nitrogen gas at 37°C and reconstituted in absolute (99.7% anhydrous) ethanol to their original serum volumes. The extracts were stored at - 20°C until the assay was performed.

2.4.7.5.2 Preparation of the standard curve

A new standard curve was prepared for each assay to quantitate the unknown serum steroid concentrations. The standard curve contained tubes for: Total counts (TC, amount of radioactive isotope added to each of the assay tubes), Nonspecific binding (NSB, extra binding that is not related to the antibody - antigen binding), Maximum binding (B_O, amount of steroid antibody - antibody interaction in the assay) and seven standard concentrations of steroid (5 pg, 10 pg, 25 pg, 50 pg, 100 pg, 300 pg, and 500 pg).

2.4.7.5.3 Steroid assay procedure

The tubes containing the standard curve concentrations, the commercial control extracts (100 µL) and the rat extracts (200 µL for estradiol[1], 100 µL for progesterone and testosterone) were evaporated to dryness in a fumehood overnight.

The assay tubes were placed on trays and were kept cold by keeping them in a larger container filled with ice. PBS, ^3H estradiol, progesterone, or testosterone tracer, and antibody

[1] to ensure that the unknown rat sera estradiol concentrations could be measured on the linear portion of the standard curve, an extra 100 µL of rat serum extract was used and the final concentration results were divided by 2.

were then added as indicated in Table 4a. The tubes were covered with parafilm, gently vortexed and incubated for 20 h at 4°C.

The bound antibody was separated from the unbound antibody, by adding 500 µL of a dextran-charcoal mixture (25 mg dextran T70, 250 mg carbon decolorizing agent in 100 mL PBS, which had been mixed overnight at 4°C) to all tubes except the TC. The tubes were incubated at 4°C (15 min for estradiol, 25 min for progesterone and 20 min for testosterone) and then centrifuged at 3,000 rpm, at 4°C for 15 min. The supernatant was decanted into plastic scintillation vials and 3 mL of scintillation cocktail added. The vials were vortexed and 6 - 8 hours later, each vial was counted for 60 seconds on a Betascintillation counter.

Table 4a. Tubes and the amount of reagent required for the preparation of the steroid standard curve

Tubes in Triplicate	Purified steroid 1 ng/mL	RIA Buffer	^{3}H estradiol, progesterone, or testosterone Tracer (≈10,000 cpm /tube)	Steroid Antibody	Dextran Charcoal
Total Count (TC)	-	700 µL	100 µL	-	-
Nonspecific Binding NSB	-	200 µL	100 µL	-	500 µL
Antibody binding	-	100 µL	100 µL	100 µL	500 µL
5 pg	5 µL	100 µL	100 µL	100 µL	500 µL
10 pg	10 µL	100 µL	100 µL	100 µL	500 µL
25 pg	25 µL	100 µL	100 µL	100 µL	500 µL
50 pg	50 µL	100 µL	100 µL	100 µL	500 µL
100 pg	100 µL	100 µL	100 µL	100 µL	500 µL
300 pg	300 µL	100 µL	100 µL	100 µL	500 µL
500 pg	500 µL	100 µL	100 µL	100 µL	500 µL

2.4.7.5.4 Quality control of steroid assay

The cross-reactivity of the testosterone antisera with other androgens was substantial and therefore steroids measured using this antisera were referred to as androgens.

The RIA results from the assay were accepted when:

1. the antibody binding was between 40 - 60 %. This ensured that there were optimal conditions for specificity and sensitivity in the assay. This value was obtained by comparing the cpm in the B_0 tube to the cpm in the TC tubes and multiplying by 100 (ie. estradiol [B_0 (mean 3925.7) ÷ (TC (mean 8915.7)] x 100 = 44%)

2. the commercial control samples were ± 2 standard deviations of the manufacturer reference means and previously run mean reference ranges, and

3. the non-specific binding in the assay was less than 5%. This value was obtained by comparing the cpm in the NSB tubes to the cpm in the TC tubes and then multiplying by 100 (ie. Estradiol [NSB (mean 174) ÷ (TC (mean 8915.7)] x 100 = 2 %).

The coefficient of variation is defined as the sample variability relative to the mean of the sample (ie. CV = [(standard deviation) ÷ (mean)] x 100). The intra-assay (difference within one assay) CV's were obtained by using extracts of normal rat sera (duplicate tubes assayed near the beginning of the assay and duplicate tubes from the same rat extract, assayed near the end of the assay) (Table 4b). The inter-assay (difference between several assays) CV's were determined by using the same rat extract for many different RIA's, after the elapse of various different periods of time (days or months) (Table 4b).

Table 4b. Example calculation results for progesterone inter-assay and estradiol intra-assay coefficient of variation

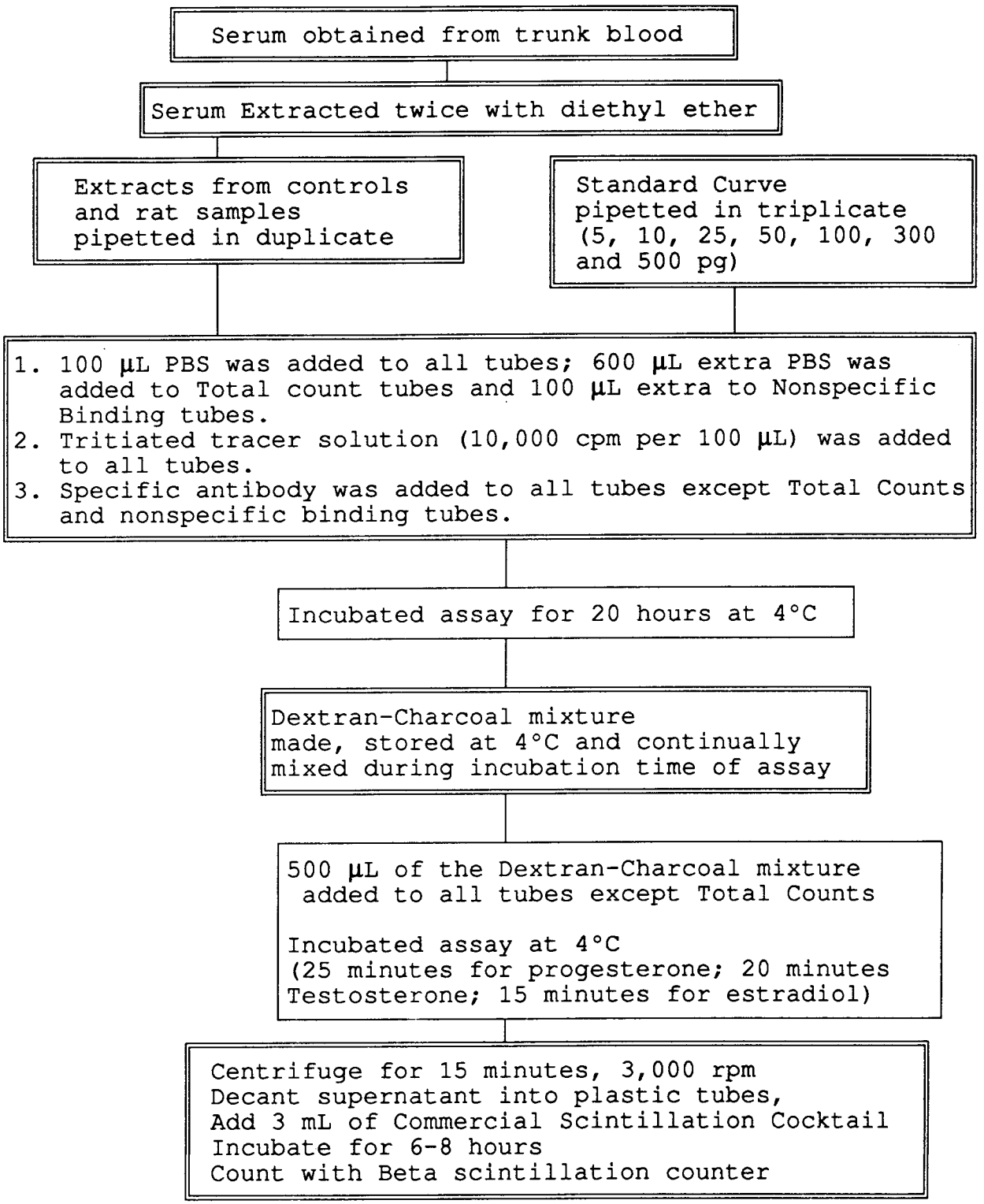
Inter-assay variation		Intra - assay variation	
Progesterone February 3, 1991	8.47 ng/mL	Estradiol Mean February 7, 1991 (beginning of assay)	147 pg/mL
Progesterone February 7, 1991	7.46 ng/mL	February 7, 1991 (end of assay)	156 pg/mL
Progesterone June 15, 1991	8.72 ngmL		
Mean: Standard deviation:	8.50 ng/mL 0.79 pg/mL		151 pg/mL 6.4 pg/ mL
Coefficient of Variation:	9.3 %		4 %

The intra- and interassay coefficients of variation were 5% and 7% for estradiol, 8% and 9% for progesterone and 5% and 6% for androgens, respectively, using low and high concentration Tri-Rac-R-Dade control extract pools. The limits of sensitivity calculated as the 95% confidence limit of the B_0 tubes was 10.2 pg/mL for estradiol, 12.5 pg/mL for progesterone and 17.7 pg/mL for androgens (Table 4c).

Table 4c. Example calculation of RIA limits of sensitivity

Limits of sensitivity: Estradiol (cpm)	
B_0 cpm = 3959, 3767, 4011, 3819 mean = 3889; 1 standard deviation = 115 cpm bottom limit (2 std dev.) = 3889 - (2 x 115) = 3659	Standard Curve cpm 5 pg = 3840 10 pg = 3581 25 pg = 3521
In this example; the limit of detection:	< 10 pg but > 5 pg

Figure 3. Flowchart for the radioimmunoassay procedure



2.4.7.6 Data analysis

The ovulatory response, uterine and ovarian weight (corrected to 100 g rat body weight), serum steroid concentrations (estradiol, progesterone and androgens), and ratio of the uterine luminal/outer uterine perimeters were expressed as a mean ± standard deviation for each group at each time and were evaluated statistically by using the student t-test. The data was independent at each time, therefore multiple comparisons were not done. Comparisons at $p<0.05$ were considered to be statistically different (Zar, 1984).

The steroid hormones concentration and morphological assessments were also analyzed by contingency tables where the groups of 4 IU PMSG treated immature rats, 40 IU PMSG treated immature rats and mature rats were compared to one another as well as within each group. The times 0, 24, 48, 72 and 96 h were placed in one stage ("pre") and groups at times 120, 144, 168, 192 and 216 h were placed in another stage ("post"). In the mature rats, proestrus and estrus were placed in one stage ("pre") and metestrus and diestrus were placed in another stage ("post"). These times and stages were chosen to correspond to the relative differences in the serum concentration of progesterone as well as the physiological event of ovulation (lower concentration of progesterone and preovulatory and ovulation in progress versus higher concentration of progesterone and completed ovulation) (Table 5a).

2.4.7.6.1 Amount of the glycocalyx and secretions

The histochemical data for all the carbohydrate staining methods were originally graded 0 - 4 for the relative area (amount) of the glycocalyx and free secretions (Table 5b). The values were statistically analyzed after reducing the number of grades from four to two. (4 (maximum), and 3 (moderate) = 2 ; while 2 (some), 1 (trace), 0 (not present) = 1

2.4.7.6.2 Colour of the staining of the glycocalyx and secretions

The histochemical staining data for the analysis of the differences in colour for the ratio of sialic acid:neutral sugars were originally graded 1 - 7 but were recoded so that values ≤ 3 were now given a value of 1 (sialic acid) and values > 3 were then given a value of 2 (neutral sugars) (Table 5b). The method for the detection of the ratio of sulphate:sialic acid, originally graded 1 - 9 were recoded so that values > 4 were given a value of 2 (sialic acid) and values ≤ 4 were given a value of 1 (sulphate) (Table 5b). The method for the detection of the ratio of sulphate esters:neutral sugars was originally graded 1 - 8 and the values were recoded so that the values > 4 were given a value of 2 (neutral sugars) and values ≤ 4 were given a value of 1 (sulphate) (Table 5b). The grades were then statistically analyzed with 2 x 2 x 3 (ie. 4 IU PMSG group versus 40 IU PMSG group versus mature rats) and 2 x 2 contingency tables (ie. 4 IU PMSG group versus 40 IU PMSG group) using the Fisher Exact Test (Two-tailed). Comparisons with the 2 x 3 contingency table were considered statistically significant at $p<0.05$ but comparisons with the 2 x 2 contingency tables were considered statistically significant at $p<0.001$ (Zar, 1984).

Table 5a. Summary of the combination of rat groups for statistical analysis of the histochemical results

Group	Hours included in "pre"	Hours included in "post"
4 IU PMSG treated immature rats	0, 24, 48, 72, 96	120, 144, 168, 192, 216
40 IU PMSG treated immature rats	24, 48, 72, 96	120, 144, 168, 192, 216
mature rats	proestrus, estrus	metestrus, diestrus

Table 5b. Summary of the combination of histochemical methods grades for statistical analysis

Method	Original Microscopic Grade	Statistically Grouped Grade
PA*/AB1.0/S	Colour 1 - 9	≤ 4 (sulphate = 1); > 4 (sialic acid = 2)
PA*/Bh/AB1.0 /PAS	Colour 1 - 8	≤ 4 (sulphate = 1); > 4 (neutral sugars = 2)
PA*/T/Bh/PAS /KOH	Colour 1 - 7	≤ 3 (sialic acid = 1); > 3 (neutral sugars = 2)
PA*S; AB1.0; PA*/Bh/PAS	Intensity/Amount 0 - 4	≤ 2 (not present, trace, some = 1); > 2 (moderate, maximum = 2)

3.0 RESULTS

3.1 Ovulatory response to PMSG treatment

Results of the ovulatory response to 4 IU PMSG and 40 IU PMSG treatment are presented in Figure 4a.

Superovulatory treatment with 40 IU PMSG induced the first ovulations as early as 24 h after injection, while in the 4 IU PMSG group, the first oocytes were not seen until 48 h. The total number of recovered oocytes was higher in the superovulated group. The mean numbers of ovulated oocytes per rat increased gradually in both groups reaching the greatest number at 72 h. Morphologically abnormal oocytes were present at 48 h and 72 h in the 40 IU PMSG group.

3.2 Ovarian weight

The results of the wet ovarian weight corrected for body weight, in mature rats and 4 IU PMSG and 40 IU PMSG treated immature rats are shown in Figures 4 b & c.

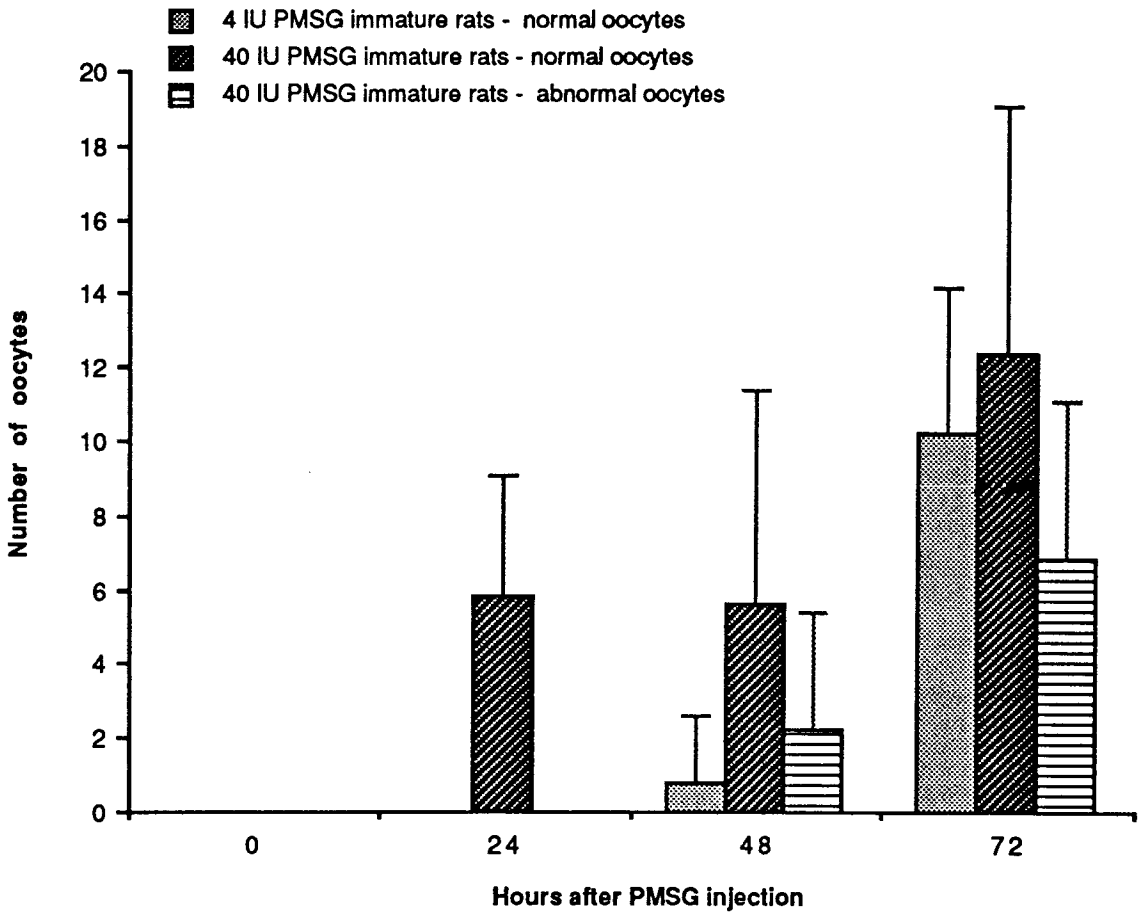
There were no differences in wet ovarian weight between the 4 IU PMSG and mature groups ($p<0.05$). The ovarian weights in the 40 IU PMSG group were greater then those from the 4 IU PMSG groups and mature groups in the "post" stage ($p<0.05$).

3.3 Uterine weight

The results for the wet uterine weight corrected for body weight, in mature rats, 4 IU PMSG and 40 IU PMSG treated immature rats are shown in Figures 5 a & b.

The wet uterine weights in the 40 IU PMSG group were greater than that found in the 4 IU PMSG groups between 96 and 120 h ($p<0.05$). The uterine weights in the 40 IU PMSG and the mature groups were similar.

Figure 4a. Ovulatory results in 4 IU & 40 IU PMSG treated immature rats[2]



[2] The oviducts from the 4 IU and 40 IU PMSG treated immature rats were flushed and oocytes counted, by Dr. Jun Young Hur and Dr. Sai Ma.

Figure 4b. Ovarian weight corrected for body weight in mature rats

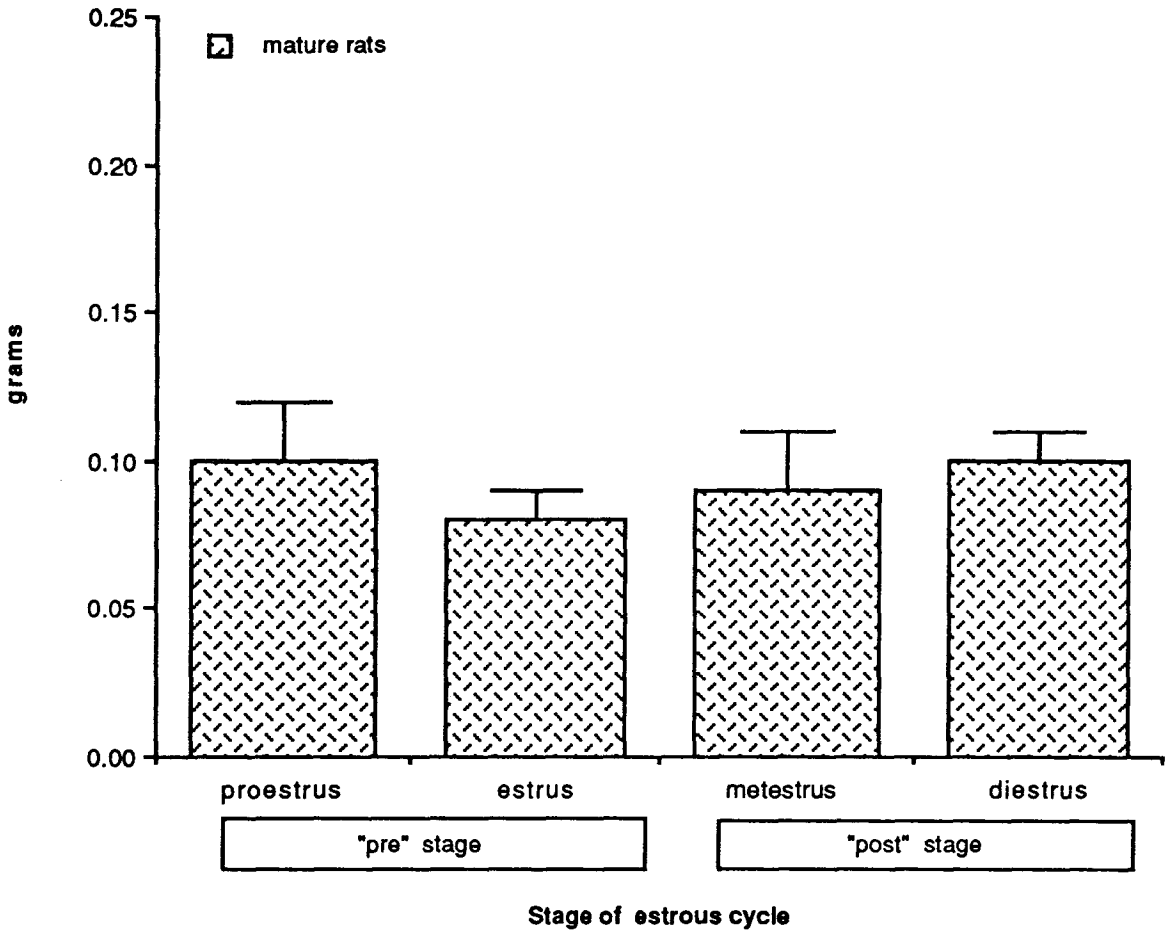


Figure 4c. Ovarian weight corrected for body weight in 4 IU & 40 IU PMSG treated immature rats

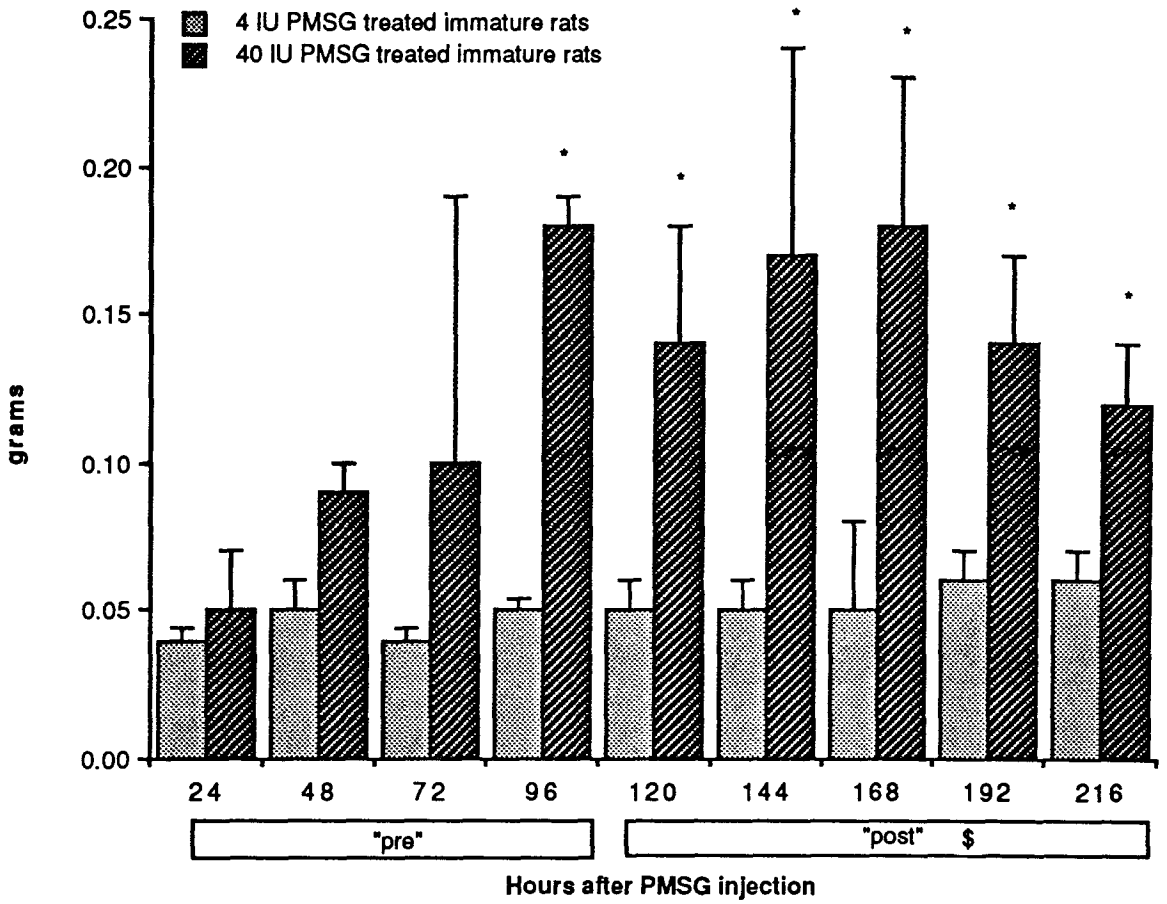


Figure 5a. Uterine weight corrected for body weight in mature rats

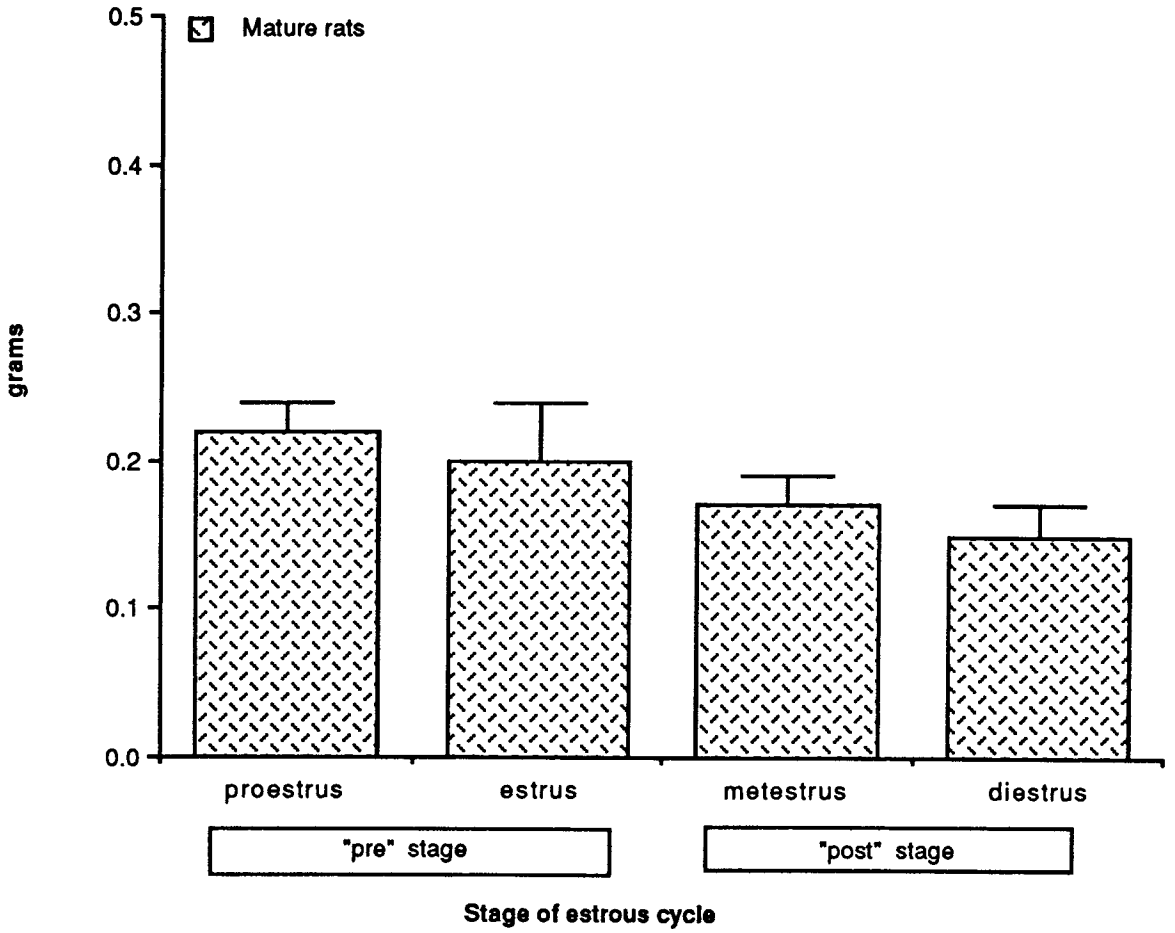
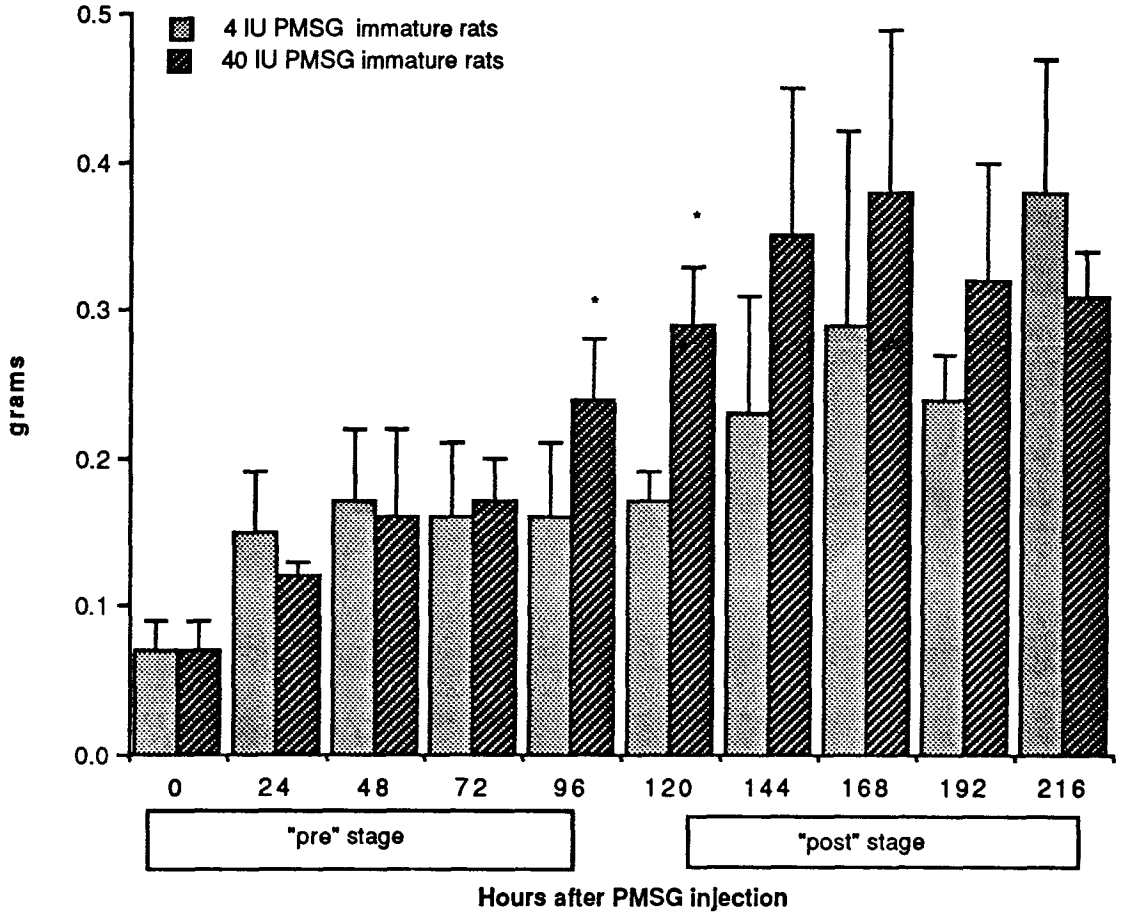


Figure 5b. Uterine weight corrected for body weight in 4 IU & 40 IU PMSG treated immature rats



* difference between groups at the same timepoint ($p<0.05$)

3.4 Steroid hormones

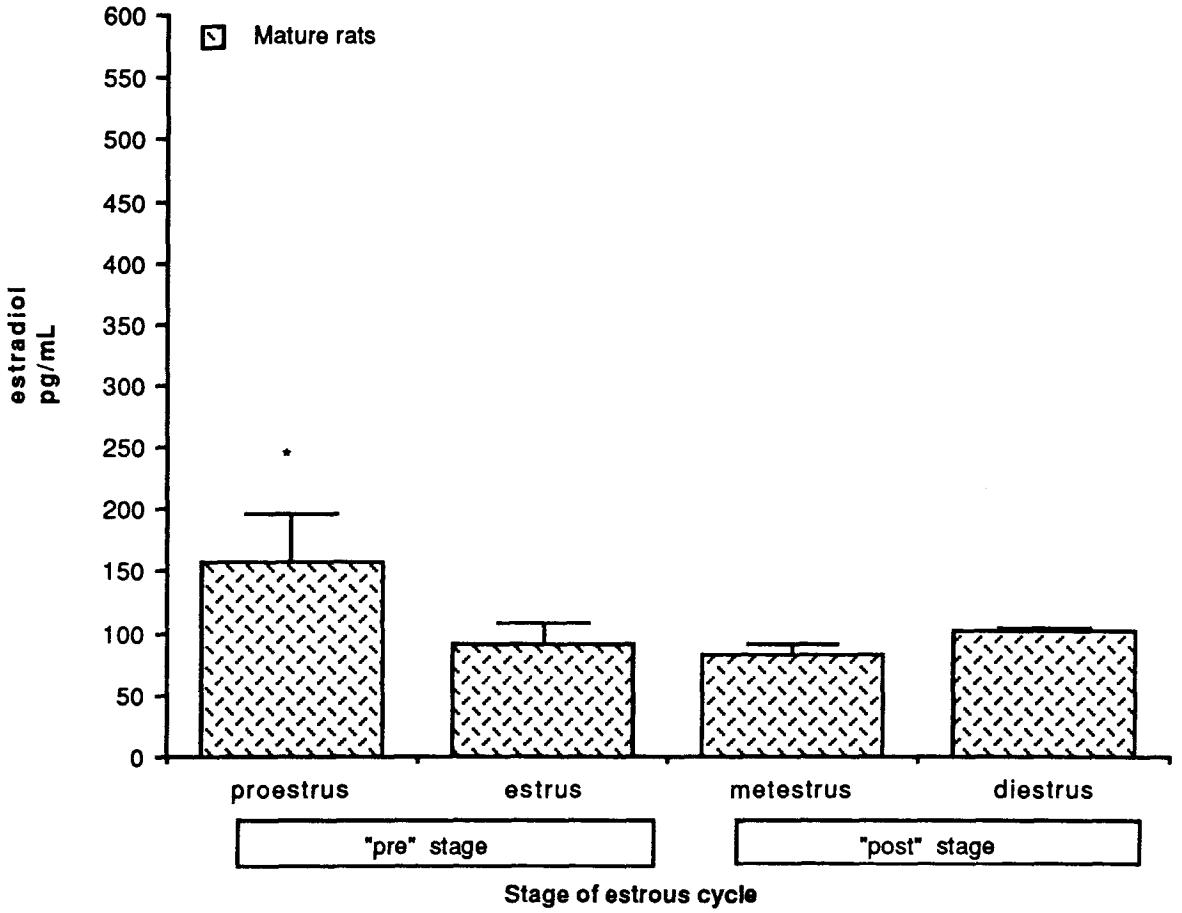
The serum steroid concentrations of estradiol, progesterone, P/E ratio and androgens for mature, 4 IU and 40 IU PMSG treated immature rats are shown in Figures 6a through 9b.

3.4.1 Serum estradiol

Mature rats displayed a cyclic pattern of serum estradiol concentrations (Figure 6a). Serum estradiol concentrations were highest during proestrus (mean 157 ± 39 pg/mL) as compared to the other phases of the cycle ($p<0.05$). No differences in estradiol concentrations were observed between estrus (mean 91 ± 17 pg/mL), metestrus (mean 81 ± 9 pg/mL) or diestrus (mean 102 ± 2 pg/mL).

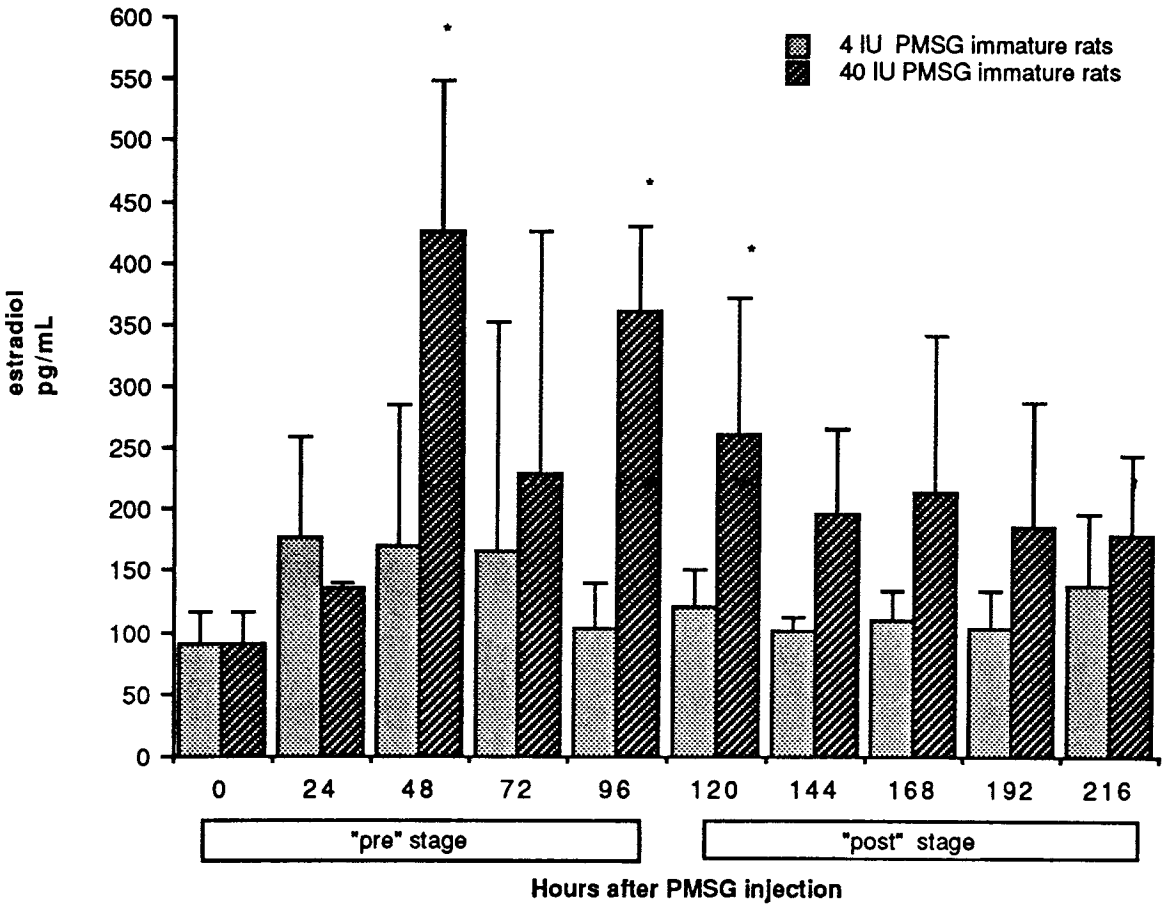
Immature rats treated with a physiological dose of PMSG (4 IU) showed a less pronounced cyclic pattern of serum estradiol concentrations (Figure 6b). Serum estradiol concentrations in the 4 IU PMSG group varied between a mean of 102 ± 11 and 176 ± 83 pg/mL which was similar to that found in mature rats. Immature rats treated with a superovulatory dose of PMSG (40 IU) had serum estradiol concentrations which varied between a mean of 136 ± 3 and 424 ± 123 pg/mL and were highest at 48 h (Figure 6b). The 40 IU PMSG group had higher serum estradiol concentrations than the 4 IU PMSG treated groups during both the "pre" and "post" stages and the mature group during estrus, metestrus and diestrus ($p<0.05$).

Figure 6a. Serum estradiol concentrations in mature rats



* difference between the groups ($p<0.05$)

Figure 6b. Serum estradiol concentrations in 4 IU & 40 IU PMSG treated immature rats



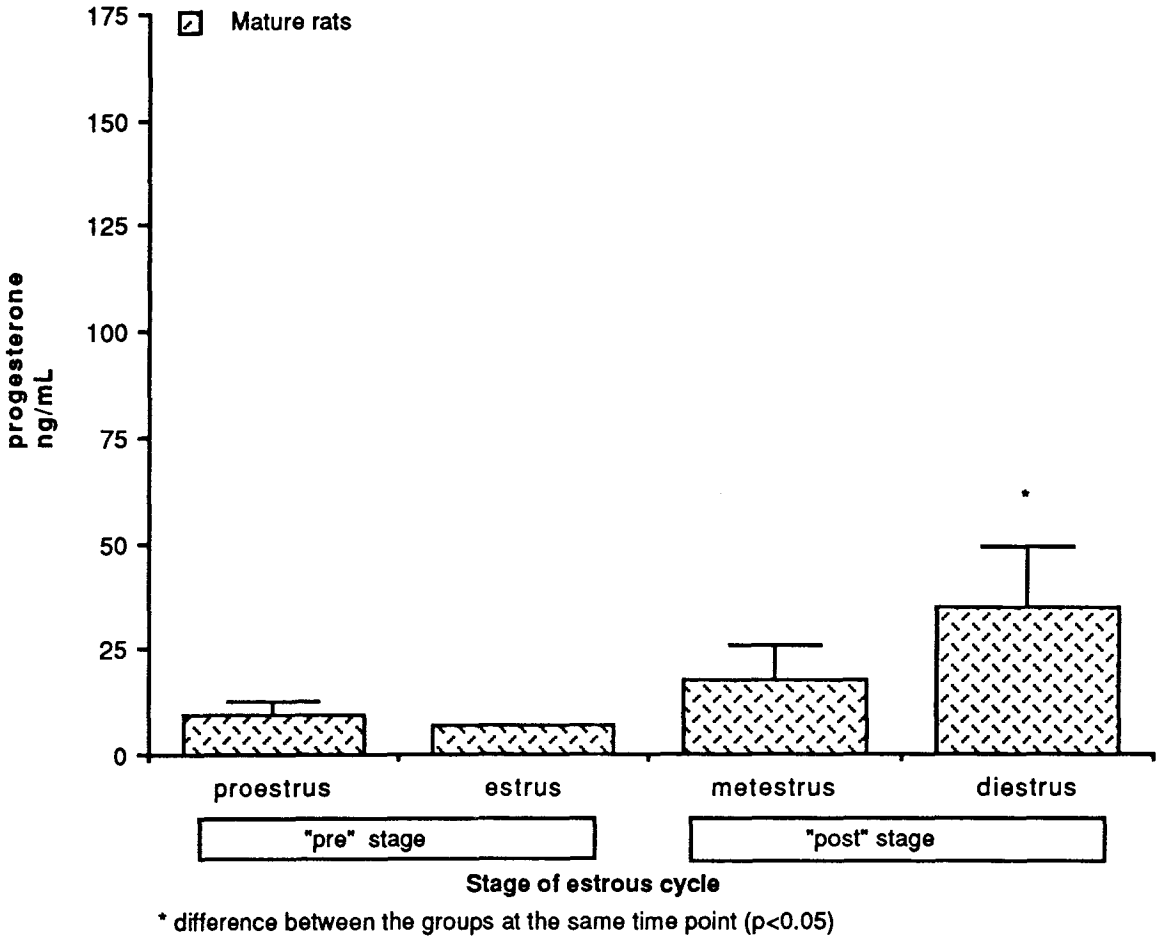
* difference between the groups at the same time point ($p<0.05$)

3.4.2 Serum progesterone

Mature rats displayed a cyclic pattern of serum progesterone concentrations (Figure 7a) which increased after (ovulation) estrus (mean 6.67 ± 0.15 ng/mL) and were highest during diestrus (mean 34.5 ± 14.4 ng/mL) ($p<0.05$). Progesterone concentrations in the 4 IU PMSG treated immature rats showed a similar pattern (Figure 7b) and ranged from a mean of 3.85 ± 1.7 ng/mL at 72 h to 45.9 ± 28.2 ng/mL at 192 h.

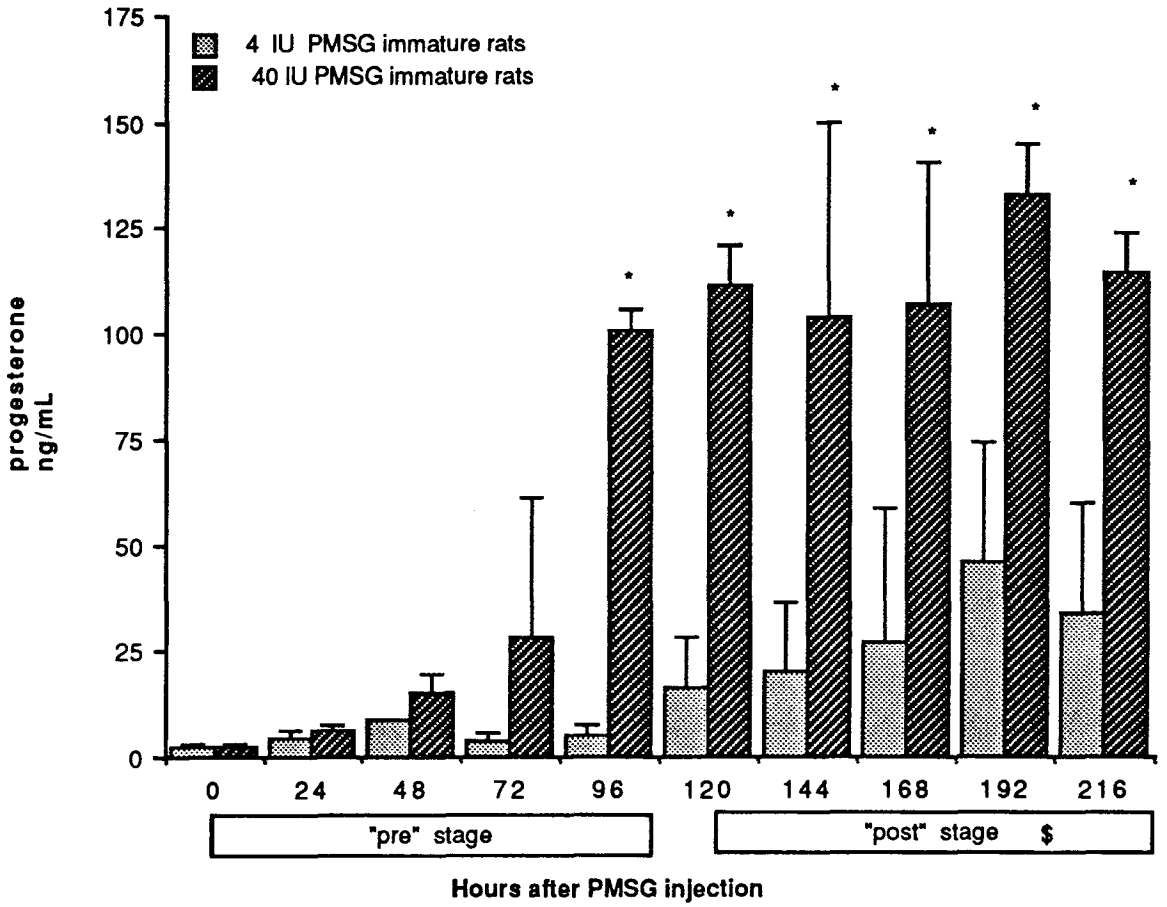
Progesterone concentrations in 40 IU PMSG treated immature rats (Figure 7b) ranged from a mean of 6.35 ± 1.5 ng/mL at 24 h to a mean of 133 ± 11.8 ng/mL at 192 h. The 40 IU PMSG group was similar to the 4 IU group during the "pre" stage. However, the serum progesterone concentrations during the "post" stage in the 40 IU PMSG treated rats were higher then that found in the "post" stage of 4 IU PMSG treated and mature rats ($p<0.05$).

Figure 7a. Serum progesterone concentrations in mature rats



* difference between the groups at the same time point ($p<0.05$)

Figure 7b. Serum progesterone concentrations in 4 IU & 40 IU PMSG treated immature rats



* difference between groups at the same timepoint ($p<0.05$)

$ difference between groups and stage ($p<0.05$)

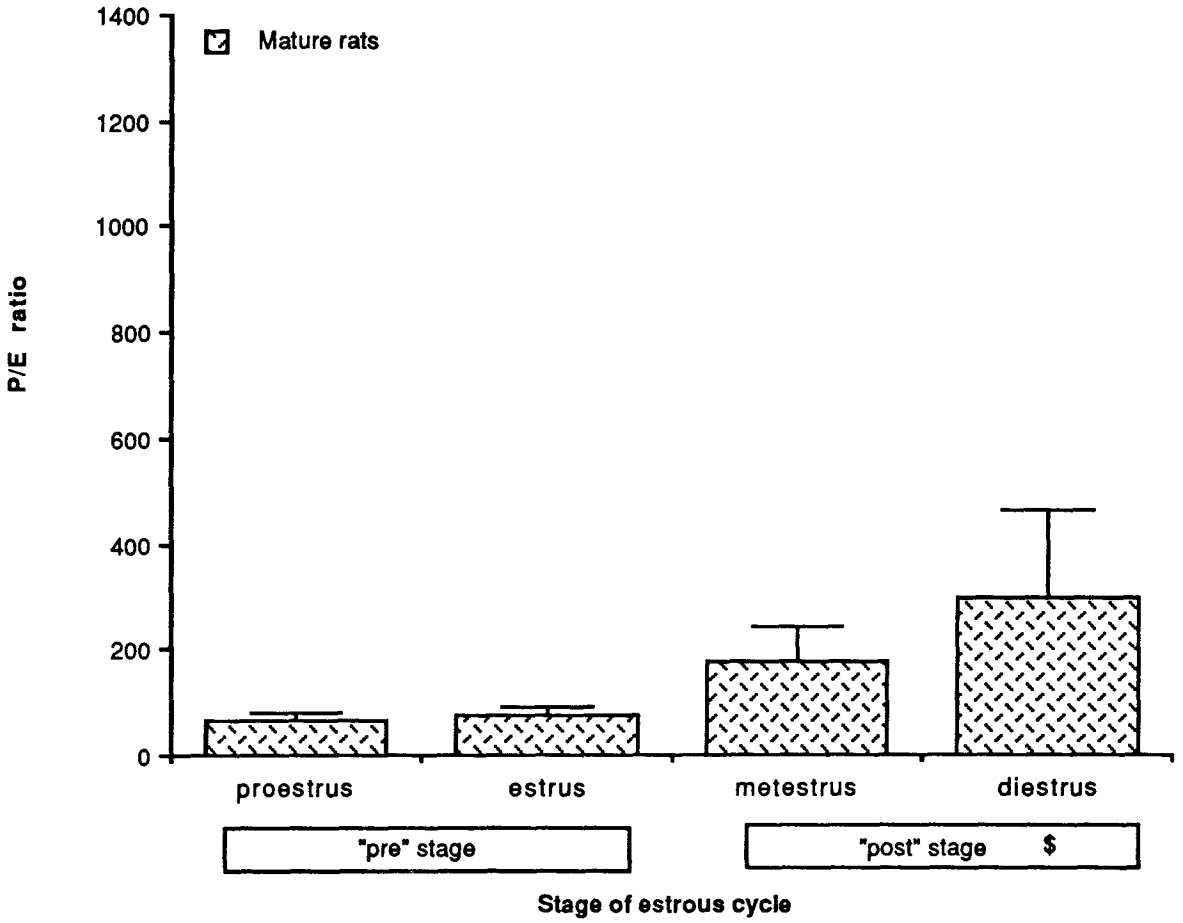
3.4.3 Progesterone-Estradiol ratio (P:E Ratio)

The P:E ratio in the mature rats (Figure 8a) was lowest at proestrus (mean 64 ± 16) and highest at diestrus (mean 295 ± 170) ($p<0.05$). The P:E ratio in the 4 IU PMSG treated rats (Figure 8b) varied from a mean of 25 ± 3 at 24 h to a mean of 453 ± 267 at 192 h ($p<0.05$) which was similar to mature rats. However, in the 40 IU PMSG treated groups (Figure 8b) the P:E ratio varied from a mean of 36 ± 1 at 48 h to a mean of 897 ± 435 at 192 h ($p<0.05$). The 40 IU PMSG treated and 4 IU PMSG treated rats had similar P:E ratios in the "pre" stage. However, the P:E ratio in the superovulated rats was higher during the "post" stage ($p<0.05$).

3.4.4 Serum androgens

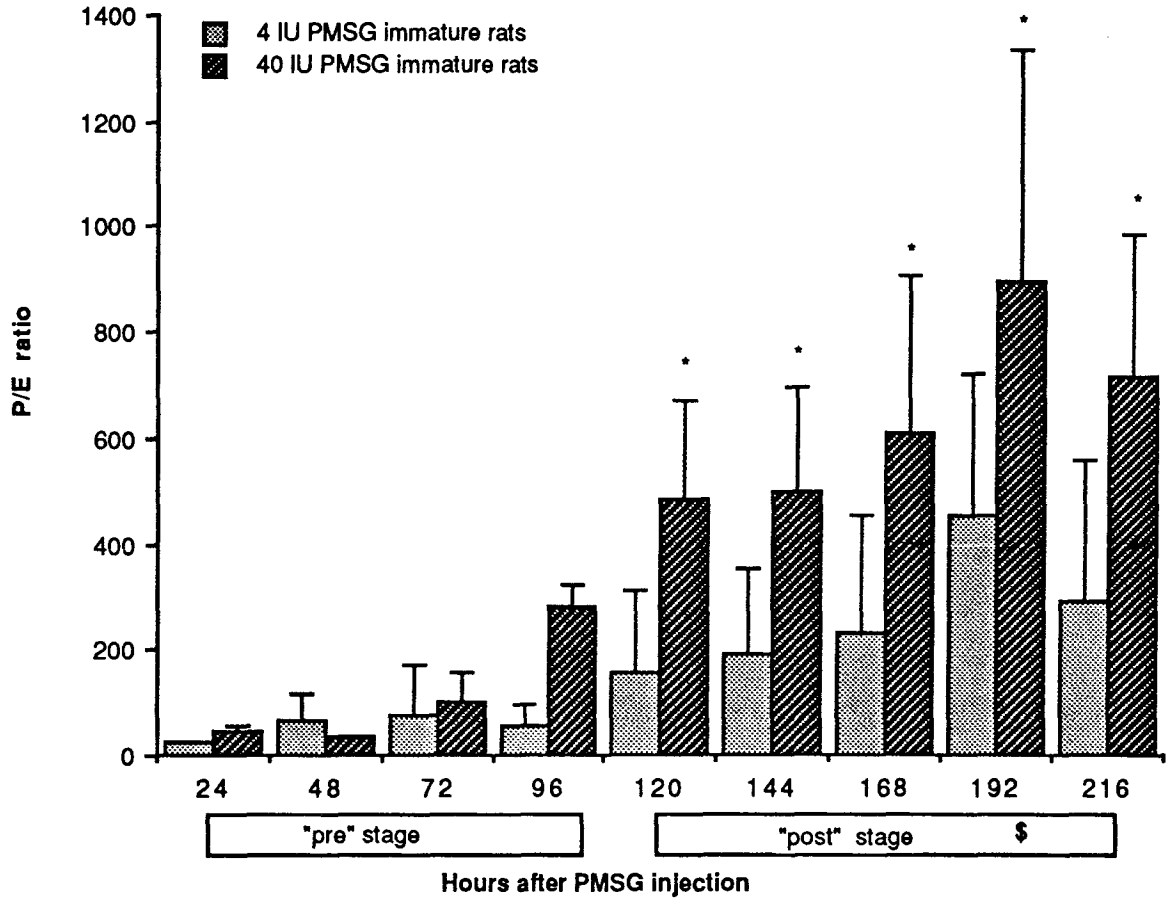
The serum androgen concentrations in mature rats (Figure 9a), varied from a mean of 0.41 ng/mL in proestrus, 0.48 ng/mL in estrus, 0.3 ng/mL in metestrus and 0.39 ng/mL in diestrus. The serum androgen concentrations in the 4 IU PMSG treated group (Figure 9b), ranged from a mean of 0.31 ± 0.22 ng/mL at 72 h to a mean of 1.92 ± 1.4 ng/mL at 24 h which was similar to the mature rats. In contrast, the serum androgen concentrations in the 40 IU PMSG treated group (Figure 9b), ranged from a mean of 0.35 ng/mL at 72 h to a mean of 3.14 ± 1.1 ng/mL at 48 h ($p<0.001$). The 40 IU PMSG group was similar to the 4 IU group during the "pre" stage. However, androgen concentrations in superovulated rats were higher during the "post" stage when compared the "post" stage in 4 IU PMSG treated immature rats and to the mature group ($p<0.05$).

Figure 8a. Progesterone:Estradiol ratio in mature rats



$ difference between stages ($p<0.05$)

Figure 8b. Progesterone:Estradiol ratio in 4 IU & 40 IU PMSG treated immature rats



* difference between groups at the same timepoint ($p<0.05$) $ difference between stages ($p<0.05$)

Figure 9a. Serum androgen concentrations in mature rats

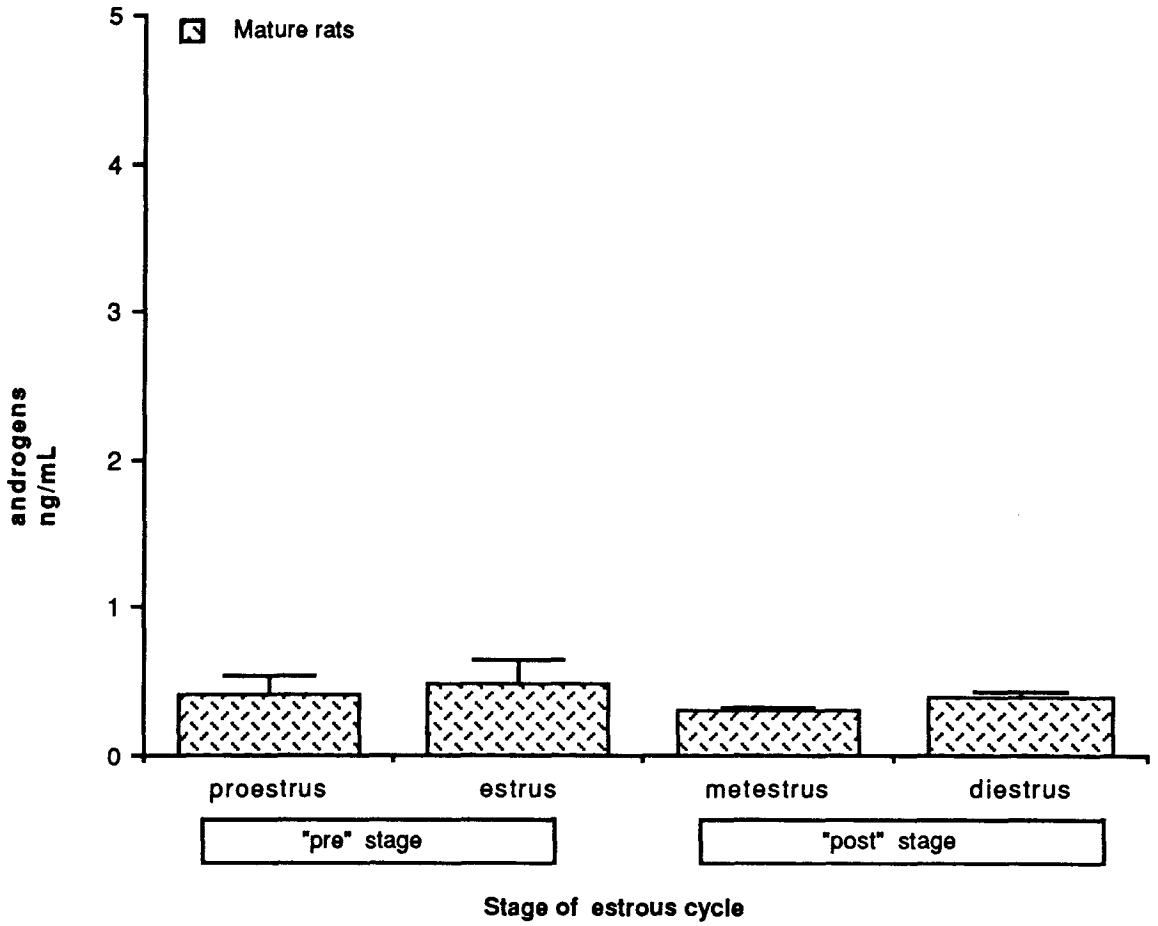
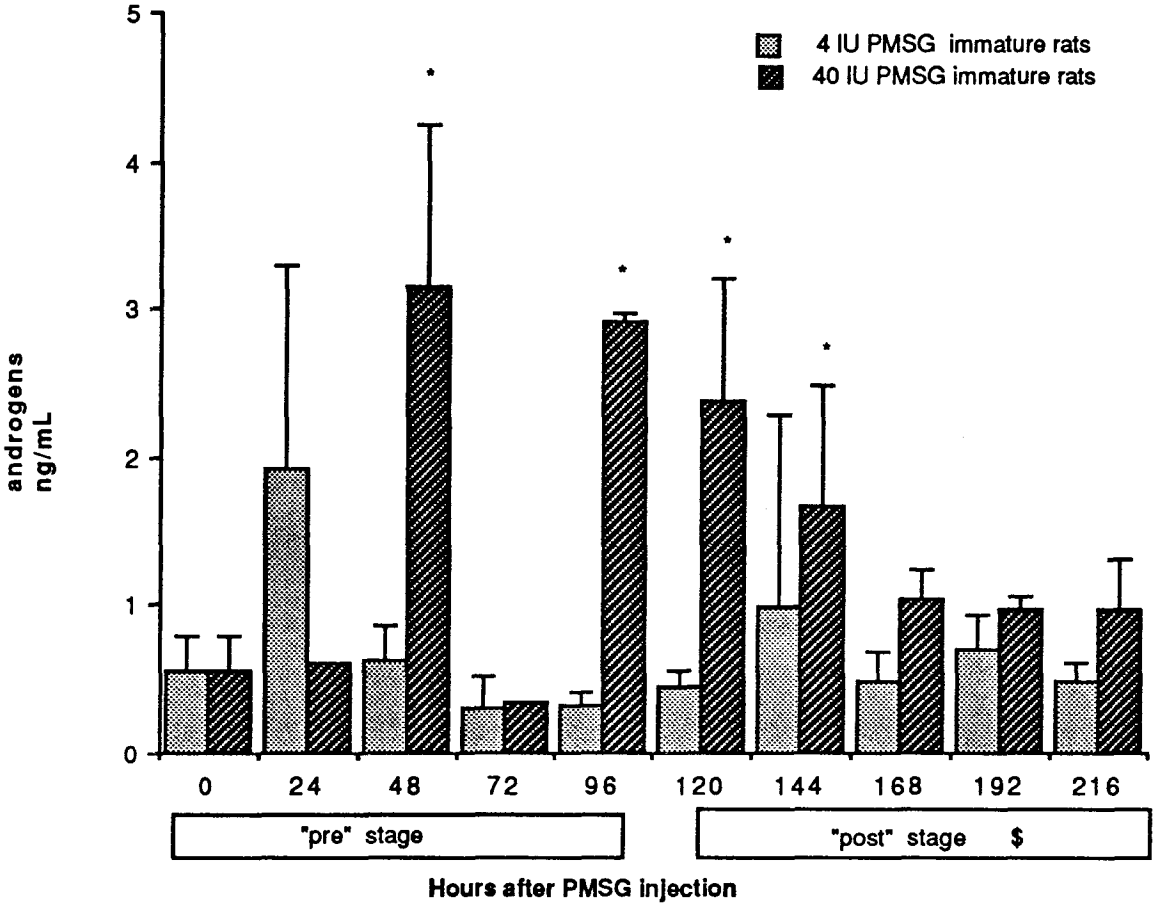


Figure 9b. Serum androgen concentrations in 4 IU & 40 IU PMSG treated immature rats



* difference between the groups at the same time point ($p<0.05$)

$ difference between groups within stage ($p<0.05$)

3.5 Histological assessments

Representative photomicrographs of the middle uterine horn cross sections of the uteri from mature rats in proestrus, estrus, metestrus and diestrus and from 4 IU PMSG treated immature rats and 40 IU PMSG treated immature rats at 48 & 168 h are shown in Figures 10 - 25. Representative photographs of duplex uteri with attached vagina from mature rats, and from 4 IU PMSG and 40 IU PMSG treated rats at 48 & 168 h are shown in Figures 26 & 27.

The uterine morphological results from mature rats are shown in Table 6. The uterine morphological results for the 4 IU and 40 IU PMSG treated immature rats and mature rats are summarized in Table 7.

3.5.1 General morphology of the endometrium

There were marked differences found in the morphology of the uteri of the 40 IU PMSG treated immature rats when compared to the 4 IU PMSG treated immature rats and the untreated mature rats. In mature rats, the morphology of the endometrium varied depending on the stage of the estrous cycle. In proestrus, the major feature was a greatly distended simple uterine lumen (Figure 10). The surface epithelium was columnar with round basal nuclei taking up half of the cell (Figure 12). Each nuclei contained 2 to 3 nucleoli. There was moderate granulocytic infiltration of the endometrium, with a rare mitotic figure seen in the surface epithelium and subepithelial stroma. The glands were lined with short columnar cells and the glandular lumen appeared empty. In estrus, the lumen was still open but had a few simple infolds (Figure 11). The surface epithelium consisted of packed tall columnar cells with round to oval nuclei, taking up one third of the cell (Figure 13). Each nucleus contained 1 to 2 nucleoli. There was extensive granulocytic infiltration

of the endometrium, with occasional mitotic figures seen in the surface epithelium and subepithelial stroma. The glands were lined with short columnar epithelial cells which showed evidence of vacuolation. In metestrus, the uterus appeared similar to estrous, but with more vacuolation of the surface epithelium (Figures 14 & 16). In diestrus, the lumen was simple and slit-like (Figure 15). The surface epithelium consisted of packed short columnar cells containing oval speckled nuclei taking up one half to three quarters of the cell area (Figure 17). There was moderate granulocytic infiltration of the endometrium, with a few mitotic figures seen in the surface epithelium and subepithelial stroma. In diestrus, there were many glands, which were lined with short columnar cells with nuclei containing prominent nucleoli.

In the immature rats treated with 4 IU PMSG, the morphology of the uterine endometrium showed cyclic variations similar to that found in mature rats. At 28 days of age, 0 h after injection with 4 IU PMSG, the uterine lumen was simple and slit-like. The surface epithelium was short columnar containing round nuclei, taking up half of the cell. Each nucleus contained one or two nucleoli. There was minimal granulocytic infiltration of the endometrium. The glands were lined with short columnar epithelial cells and the glandular lumen appeared empty. By 72 hours post 4 IU PMSG injection, the uterine lumen was open with a few simple infolds. The tall columnar surface epithelium had oval shaped nuclei containing one or two nucleoli. Some of the epithelial cells appeared pseudostratified. There was karryhetric debris and some epithelial vacuolation present. The glands were lined with short columnar epithelial cells which had round nuclei containing one or two nucleoli. There was mild infiltration of the endometrium with granulocytes.

The morphological pattern of the endometrium appeared to repeat itself 144 hours after 4 IU PMSG injection because the endometrium was similar to that found during

proestrus in the mature rat. The uterine lumen was beginning to open and had several simple infolds. The uterine surface epithelium was packed columnar with diffusely speckled nuclei taking up about half of the total cell area. There was a moderate amount of granulocytic infiltration of the endometrium. The glands were lined with cuboidal shaped epithelial cells containing round nuclei. By 168 h post 4 IU PMSG injection, the uterine lumen was open with many simple infolds (Figure 22). The packed, tall columnar surface epithelial cells contained diffusely speckled oval shaped nuclei taking up one third to one half of the total cell area (Figure 24). There was mild infiltration of the endometrium with granulocytes. The glands were lined with cuboidal epithelial cells containing round nuclei.

In the immature rats treated with 40 IU PMSG, the morphology of the uterine endometrium did not show cyclic variations similar to that found in the untreated mature rats or the 4 IU PMSG treated immature rats. Twenty four hours after injection with 40 IU PMSG, the uterine lumen was open with a few simple infolds. The packed columnar surface epithelium contained diffusely speckled round nuclei. There was mild infiltration of the endometrium with granulocytes. The glands were lined with cuboidal epithelial cells containing round nuclei. After 72 h post 40 IU PMSG injection, the lumen had both simple and complex infolds. The tall packed columnar surface epithelium contained round nuclei with one to two nucleoli. There was also some vacuolation of the epithelial cells. The short columnar glandular epithelium had round nuclei containing one to two nucleoli. By 144 hours post 40 IU PMSG injection, the uterine lumen was extremely complex with many extensive infolds. The packed columnar surface epithelium contained round nuclei taking up one third to one half of the total cell area. Each nucleus contained one or two prominent nucleoli. The glands were difficult to distinguish from the uterine lumen. After 168 h post 40 IU PMSG injection (Figure 21), the uterine lumen was still extremely complex, especially

at the anti-mesometrial side. However, the extensive infolds seen at 144 h were not as prominent. The packed columnar surface epithelium was contained round nuclei, taking up one third to one half of the total cell area. Each nucleus contained one to two nucleoli. The glands were impossible to distinguish from the uterine luminal epithelium (Figure 25). At 216 h post 40 IU PMSG injection the open uterine lumen had many simple and complex infolds. The packed short columnar surface epithelium contained round nuclei taking up one half of the total cell area. The glands were still not distinguishable.

During the "post" stage of the experiment, the uterus from the 40 IU PMSG treated immature rats did not resemble the untreated mature rats or the 4 IU PMSG treated immature rats. It was also visually noticeable that the amount of granulocytic infiltration of the uterine endometrium was less in the immature rat uteri when compared to the mature rat uteri.

Table 6. Uterine morphology in mature rats

Stage	Lumen	Uterine surface epithelial cells		Glands	
	Appearance	shape	nuclei	cell	contents
Pro	greatly distended open simple	columnar	round, 1/2 cell	short columnar	empty
Est	open, few simple infolds	packed tall columnar	round 1/3 cell	short columnar with vacuoles	some
Met	open, few simple infolds	packed tall columnar with vacuoles	round 1/3 cell	short columnar with vacuoles	some
Die	simple, slit-like	packed short columnar	oval, 3/4 cell	short columnar	rare

Table 7. Uterine morphology in 4 IU and 40 IU PMSG treated immature rats

Hour/ PMSG dose	Uterine lumen	Uterine surface epithelial cells		Glands	
	Appearance	Shape	Nuclei	Cell Shape	contents
0/ none	simple, slit-like	short columnar	round, 1/2 cell	short columnar	empty
24/ 4 IU	simple, several infolds,	short columnar	round, 1/2 cell	short columnar	empty
48/ 4 IU	open, many simple infolds	packed tall columnar	round, 1/4 cell	short columnar	empty
72/ 4 IU	open, occasional simple infolds	tall columnar many vacuoles	oval, 1/4 cell	short columnar	some
96/ 4 IU	open, occasional simple infolds	tall columnar	round, 1/3 cell	cuboidal	some
120/ 4 IU	open, occasional simple infolds	tall columnar	round, 1/3 cell	cuboidal	some
144/ 4 IU	open, several simple infolds	packed columnar	round, 1/2 cell	cuboidal	some
168/ 4 IU	open, many simple infolds	packed tall columnar	oval 1/3 cell	cuboidal	rare
192/ 4 IU	open, simple	packed columnar	oval, >1/2 cell	cuboidal	rare
216/ 4 IU	open, simple	packed columnar	oval, >1/2 cell	cuboidal	rare

Hour/ PMSG dose	Uterine lumen	Uterine surface epithelial cells		Glands	
	Appearance	Shape	Nuclei	Cell Shape	contents
24/ 40IU	open, few simple infolds	packed columnar	round, 1/2 cell	cuboidal	rare
48/ 40IU	open, more simple infolds	packed columnar	oval, >1/3 cell	cuboidal	rare
72/ 40IU	simple and complex infolds	tall packed columnar some vacuoles	oval	short columnar	rare
96/ 40IU	open with many simple and complex infolds	packed columnar many vacuoles	oval, 1/3 cell	cuboidal	rare
120/ 40IU	more complex, extensive infolds	tall packed columnar	round, >1/3 cell	unable to identify	
144/ 40IU	extremely complex with many more extensive infolds	packed columnar	round, >1/3 cell	unable to identify	
168/ 40IU	extremely complex with extensive infolds	packed columnar	round, >1/3 cell	unable to identify	
192/ 40IU	open, many simple infolds	packed short columnar	round	unable to identify	
216/ 40IU	open, many simple and complex infolds	packed short columnar	round, 1/2 cell	unable to identify	

3.5.1.1 Description of photographs

Figures 10 - 25 are representative light photomicrographs of rat uteri from the 4 IU PMSG, 40 IU PMSG treated immature rats and the untreated mature rats. The cross-sections from the middle segment of the uterine horn were stained with hematoxylin and eosin.

Figure 10. Proestrus, mature rat, 153X. Note the greatly distended simple round lumen. Note also the short distance between the uterine lumen and inner circular muscle layer of the myometrium (indicated by bar). The serum steroid concentrations in this rat were estradiol 176 pg/mL, progesterone 8.4 ng/mL, and androgens 0.55 ng/mL.

Figure 11. Estrus, mature rat, 153X. Note the simple infolds of the uterine lumen (arrowheads) and presence of numerous glands (arrows) scattered throughout the subepithelial stroma. Also note that the distance from the uterine lumen (L) to the inner circular muscle layer (cm) appears greater then that seen at proestrus (Figure 10). Ovulation occurred early in the morning of estrus. The serum steroid concentrations in this rat were estradiol 92 pg/mL, progesterone 6.8 ng/mL, and androgens 0.36 ng/mL.

Figure 10. Proestrus, mature rat, 153X.

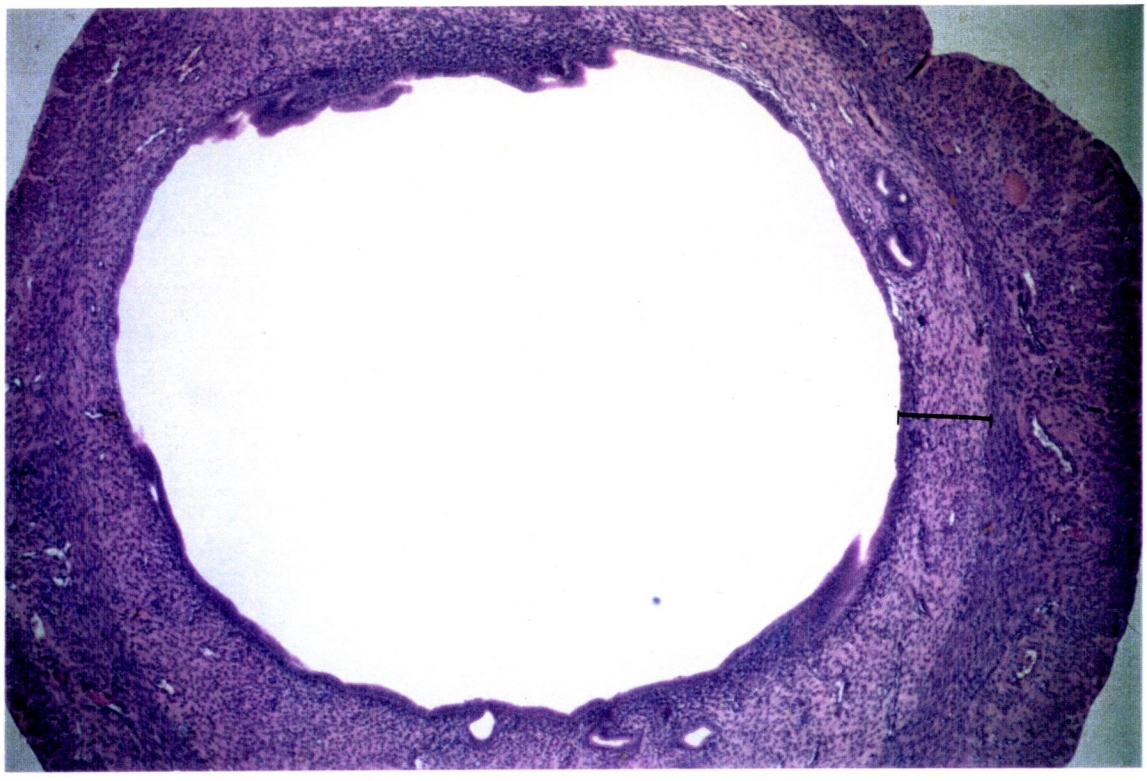


Figure 11. Estrus, mature rat, 153X.

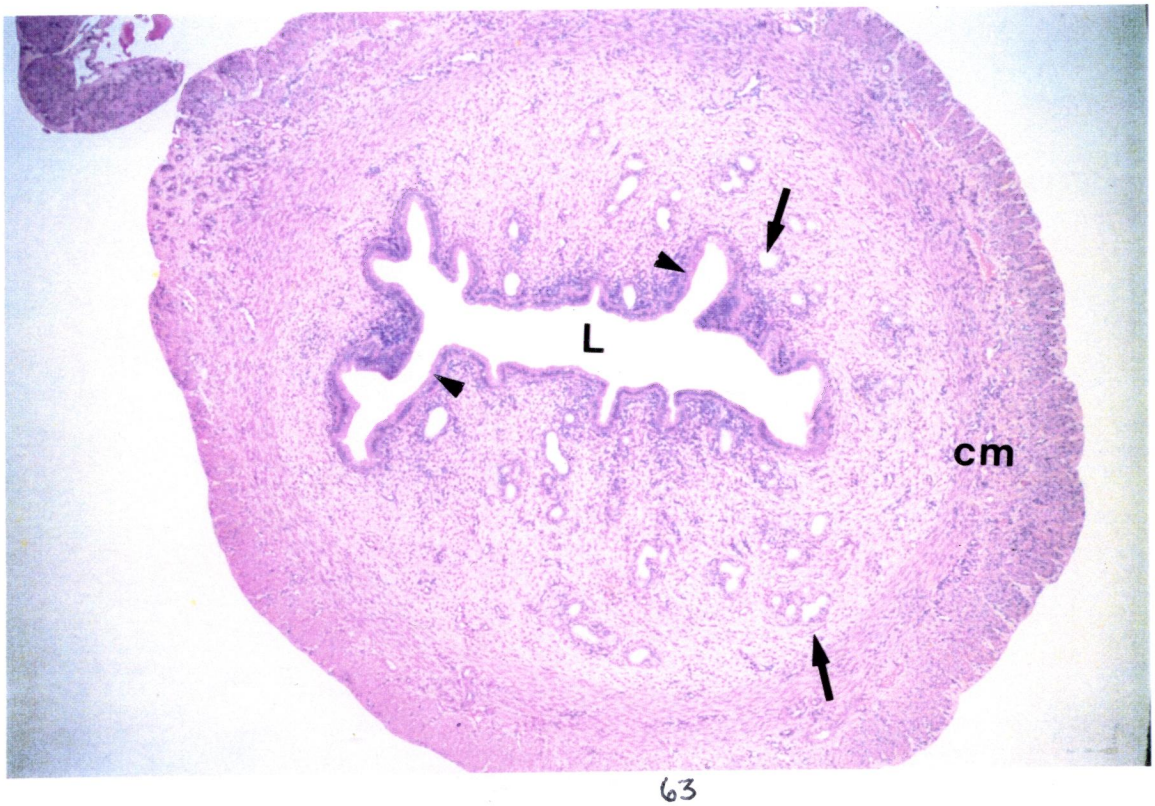


Figure 12. Proestrus, mature rat, 2472X. Note the simple layer of short columnar surface epithelial cells (arrow) which line the uterine lumen. Compare to the gland (g), which has columnar shaped epithelial cells.

Figure 13. Estrus, mature rat, 2472X. Note the packed, tall columnar surface epithelium (arrow) containing oval nuclei with prominent nucleoli. Compare the uterine luminal epithelium to the glandular (g) surface epithelium which here appears much shorter (arrowhead).

Figure 12. Proestrus, mature rat, 2472X.

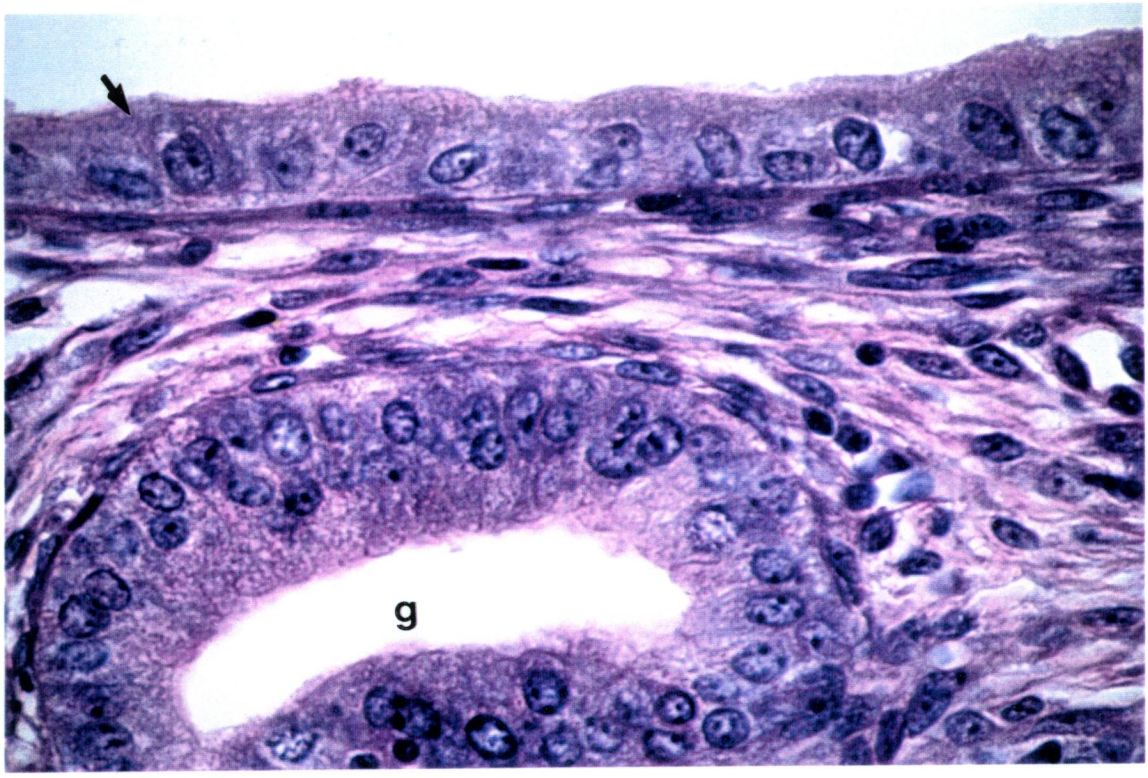


Figure 13. Estrus, mature rat, 2472X.

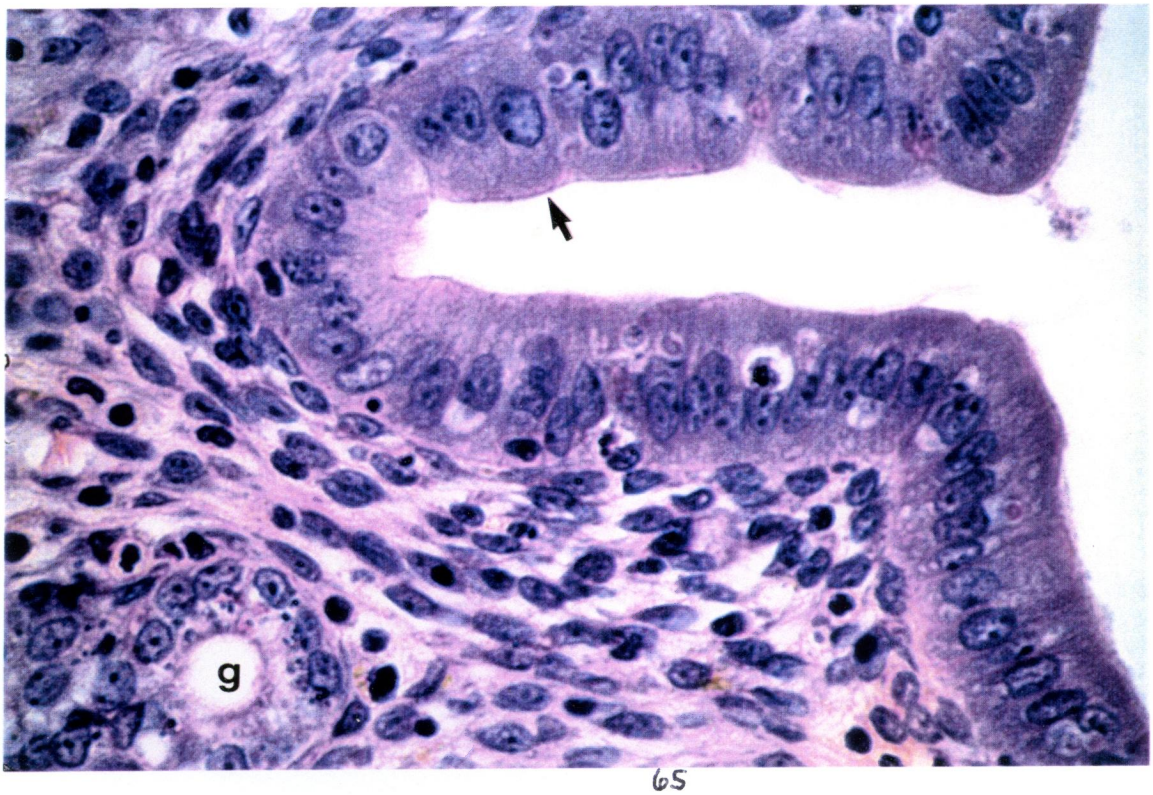


Figure 14. Metestrus, mature rat, 153X. Note the very simple infolds of the uterine lumen and notice that the glands tend to be located nearer the antimesometrial end (a) then the mesometrial end (m) of the uterus. The serum steroid concentrations in this rat were estradiol 89 pg/mL, progesterone 26.8 ng/mL, and androgens 0.33 ng/mL.

Figure 15. Diestrus, mature rat, 153X. Note the simple "slit-like" uterine lumen which runs from the mesometrial end (m) to the antimesometrial (a) end of the uterus. The glands (g) seem to be present in higher numbers at the antimesometrial end of the uterus. Also note, that the inner circular muscle layer (CM) and the muscle bundles of the outer longitudinal layer (OL) of the myometrium are distinct. The serum steroid concentrations in this rat were estradiol 100 pg/mL, progesterone 44 ng/mL and androgens 0.42 ng/mL.

Figure 14. Metestrus, mature rat, 153X.

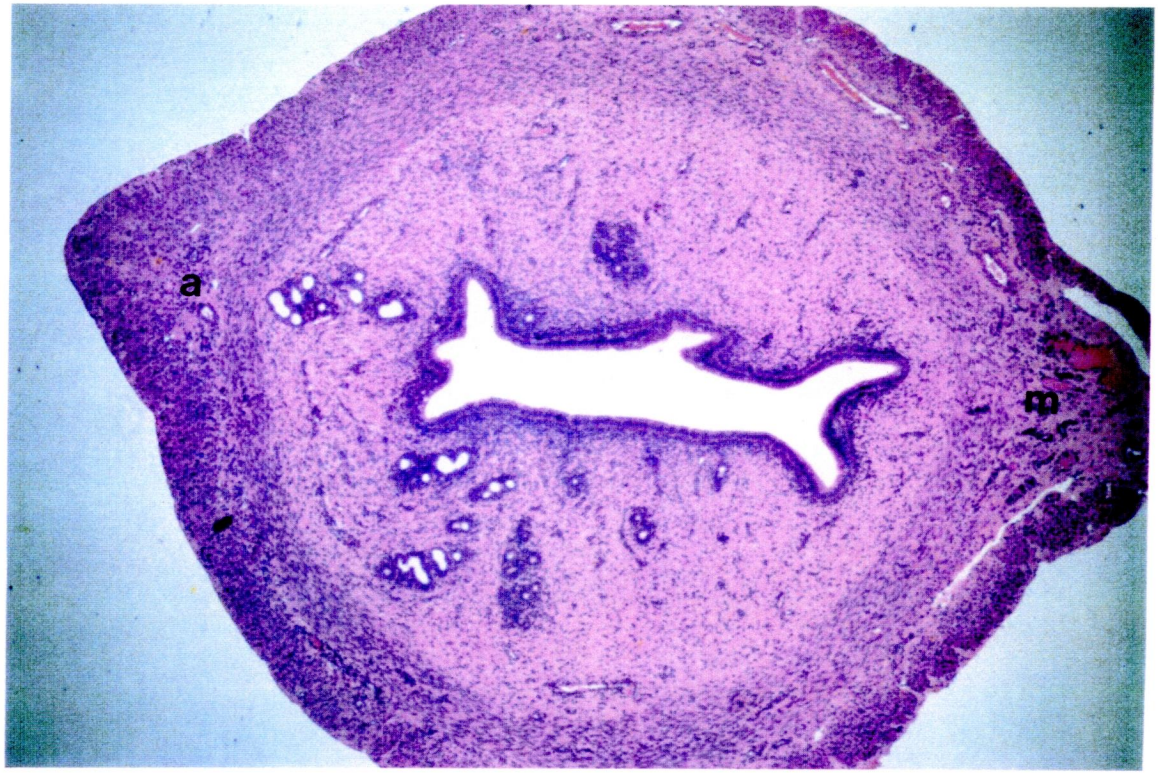


Figure 15. Diestrus, mature rat, 153X.

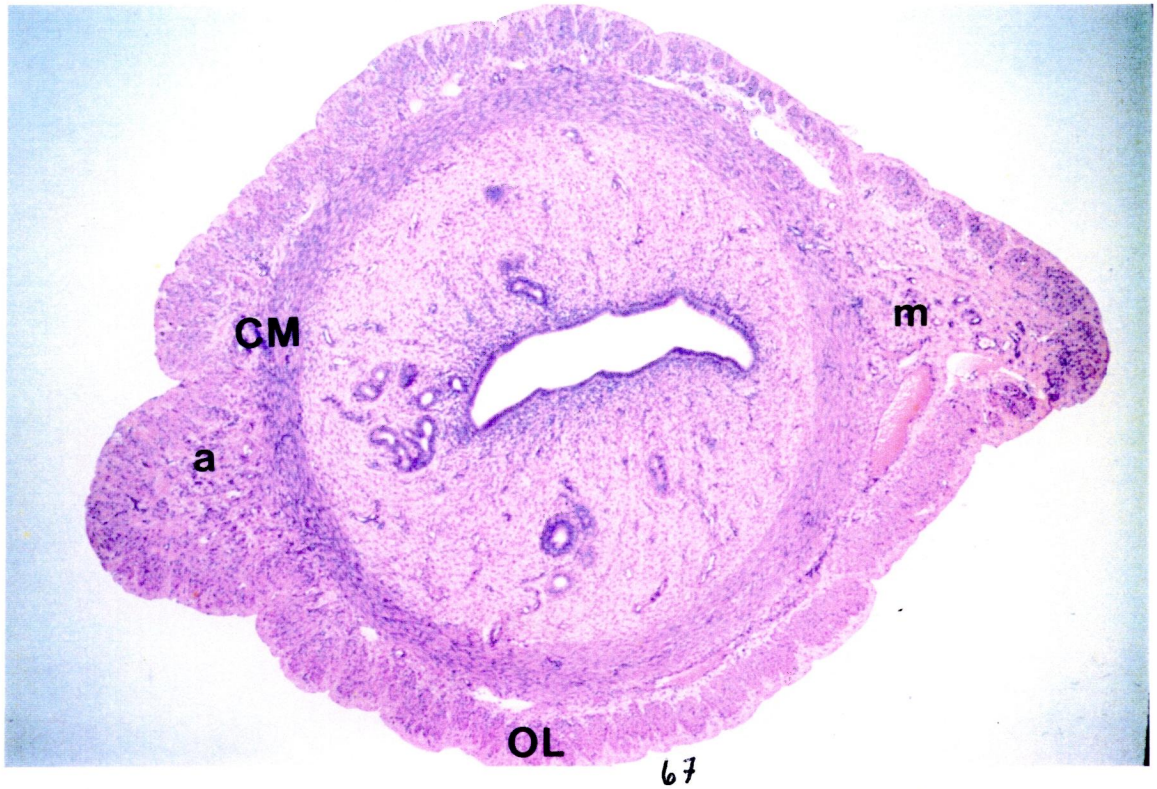


Figure 16. Metestrus, mature rat, 2472X. Note the tall columnar epithelial cells with vacuoles (arrow). Also, that the gland (g) is lined with cuboidal to short columnar epithelial cells.

Figure 17. Diestrus, mature rat, 2472X. Note the packed, columnar surface epithelial cells containing speckled nuclei (arrow) and mitotic figures (arrowhead). Also, that the gland (g) is lined with short columnar cells with a mitotic figure present (arrowhead).

Figure 16. Metestrus, mature rat, 2472X.

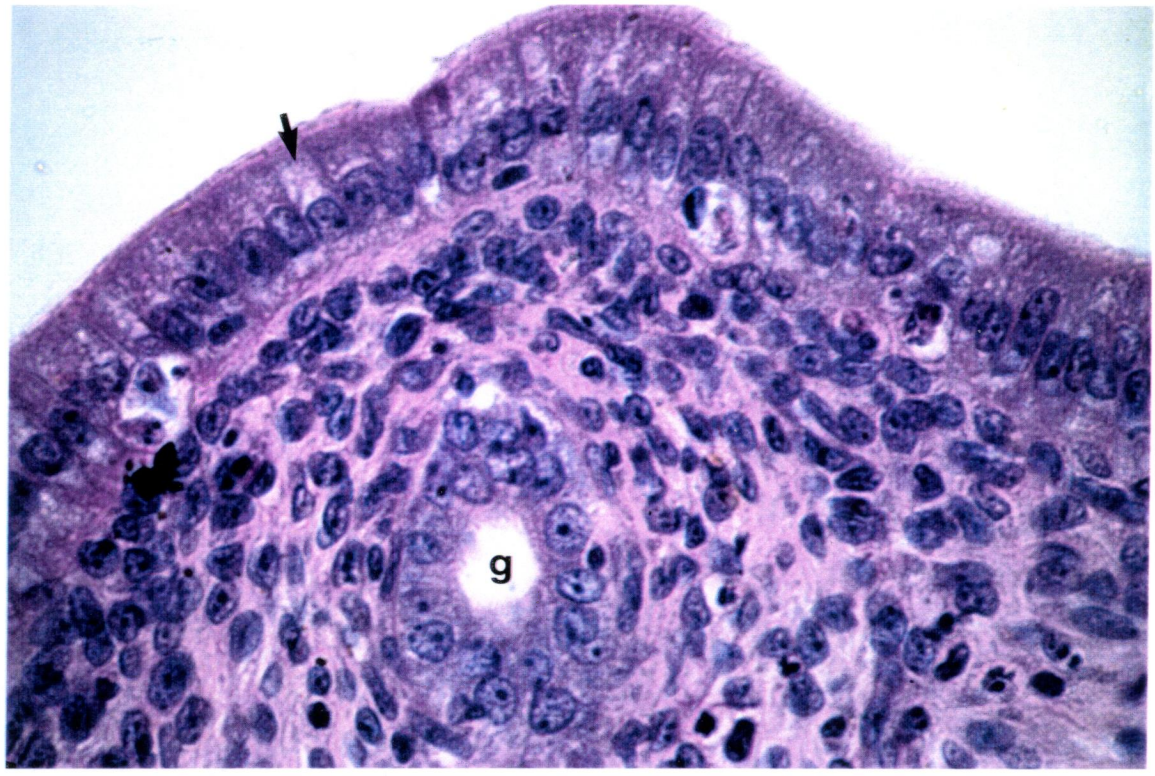


Figure 17. Diestrus, mature rat, 2472X

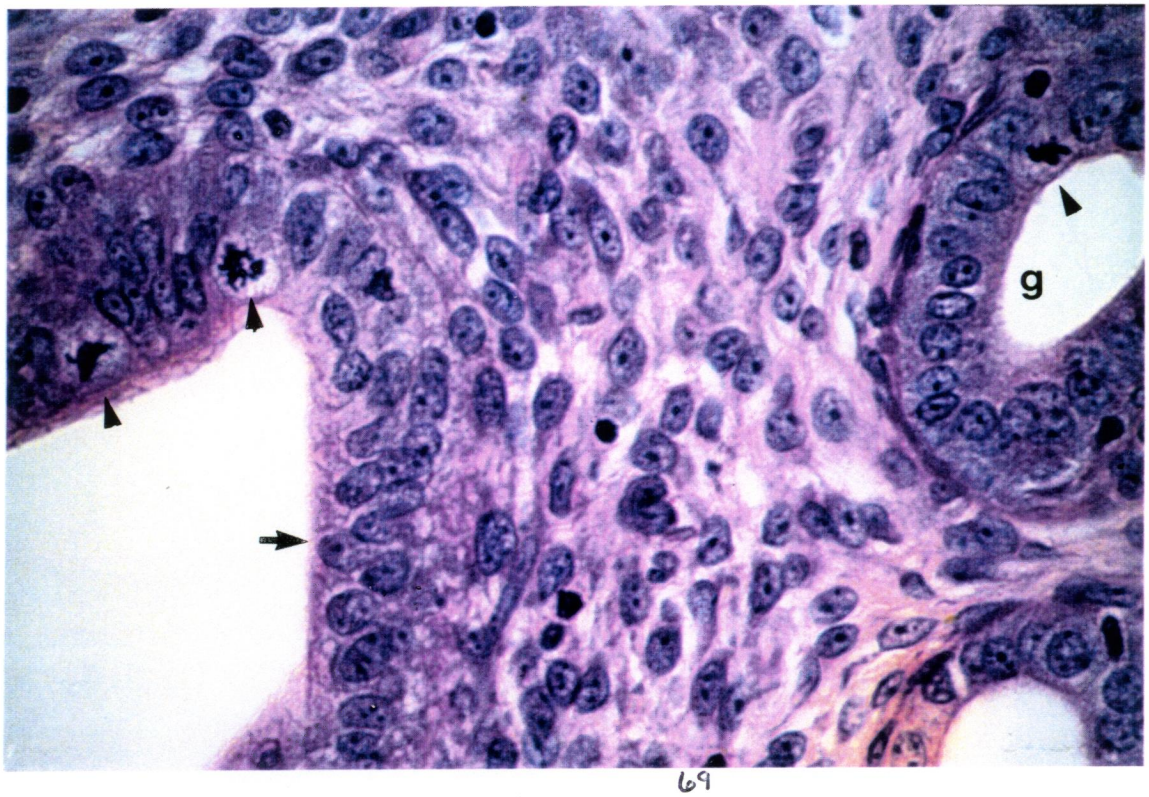


Figure 18. 30 day immature rat, 48 h post 4 IU PMSG, 153X. Note that the uterine lumen is open but has several flaccid folds (arrow), similar to that seen in estrus in the mature rat (Figure 11). There are only 2 glands (g) present in the antimesometrial region as compared to the numerous glands seen in estrus (Figure 11). Ovulation has not yet occurred. The serum steroid concentrations in this rat were estradiol 88 pg/mL, progesterone 8.8 ng/mL and androgens 0.47 ng/mL.

Figure 19. 30 day immature rat, 48 h post 40 IU PMSG, 153X. Note that the uterine lumen has several invaginations into the endometrium (arrows). Ovulation has occurred. The serum steroid concentrations in this rat were estradiol 511 pg/mL, progesterone 18.3 ng/mL and androgens 3.92 ng/mL.

Figure 18. 30 day immature rat, 48 h post 4 IU PMSG, 153X.

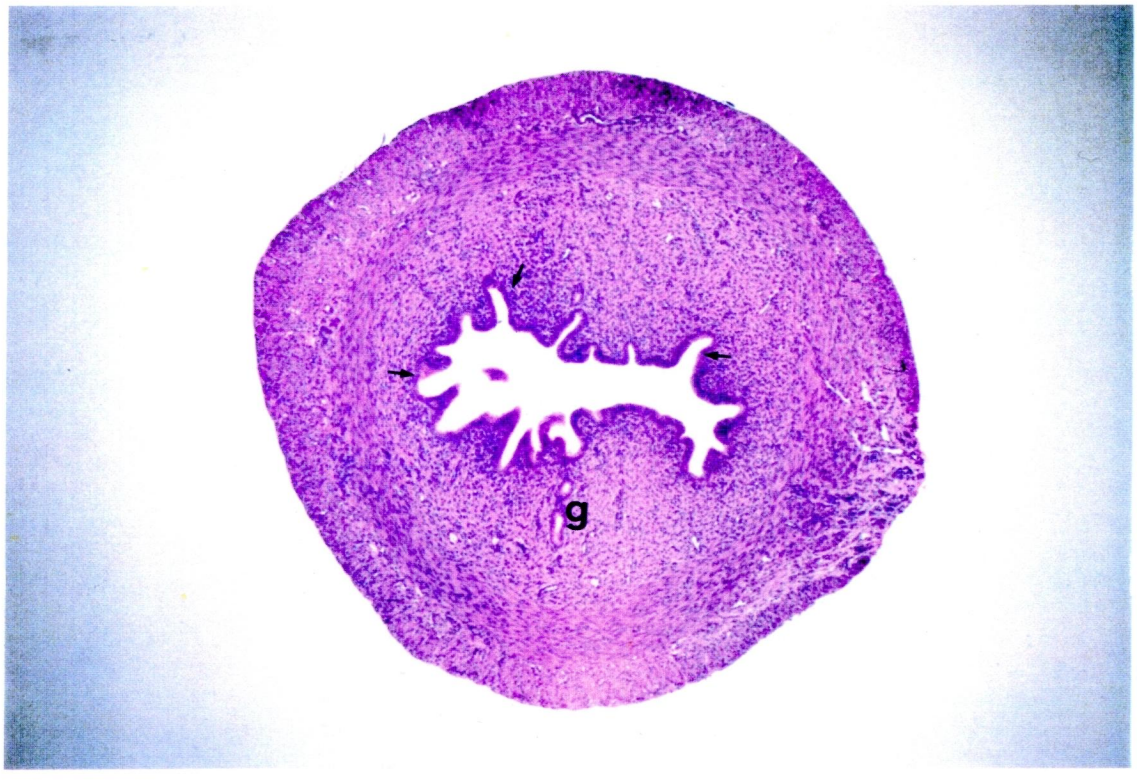


Figure 19. 30 day immature rat, 48 h post 40 IU PMSG, 153X.

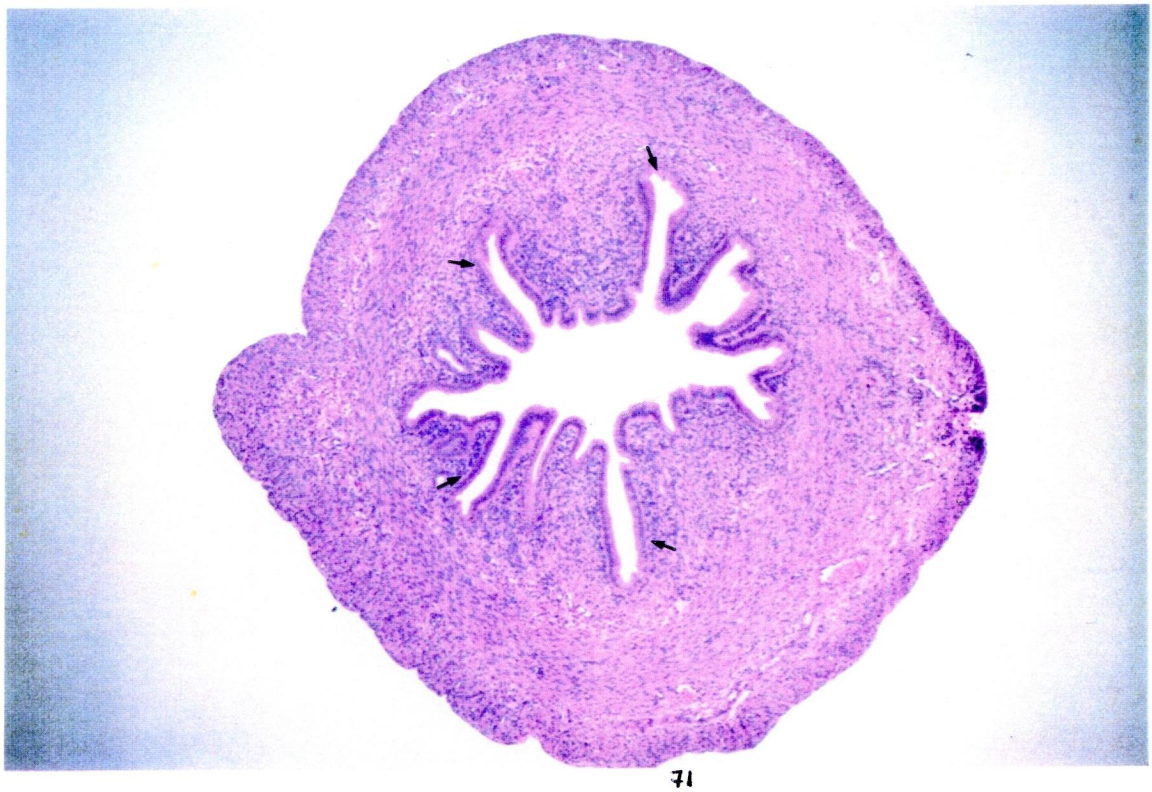


Figure 20. 30 day immature rat, 48 h post 4 IU PMSG 2472X. Note the tall, packed columnar surface epithelium containing oval nuclei with prominent nucleoli. Also note, the presence of vacuoles (arrow-v) and karyorrhectic debris (arrows) in the cytoplasm of some the surface epithelial cells.

Figure 21. 30 day immature rat, 48 h post 40 IU PMSG, 2472X. Note the packed columnar surface epithelium containing round nuclei (small arrow) with prominent nucleoli. Also note the stromal mitotic figures (arrows), indicating an actively proliferating subepithelial stroma.

Figure 20. 30 day immature rat, 48 h post 4 IU PMSG 2472X.

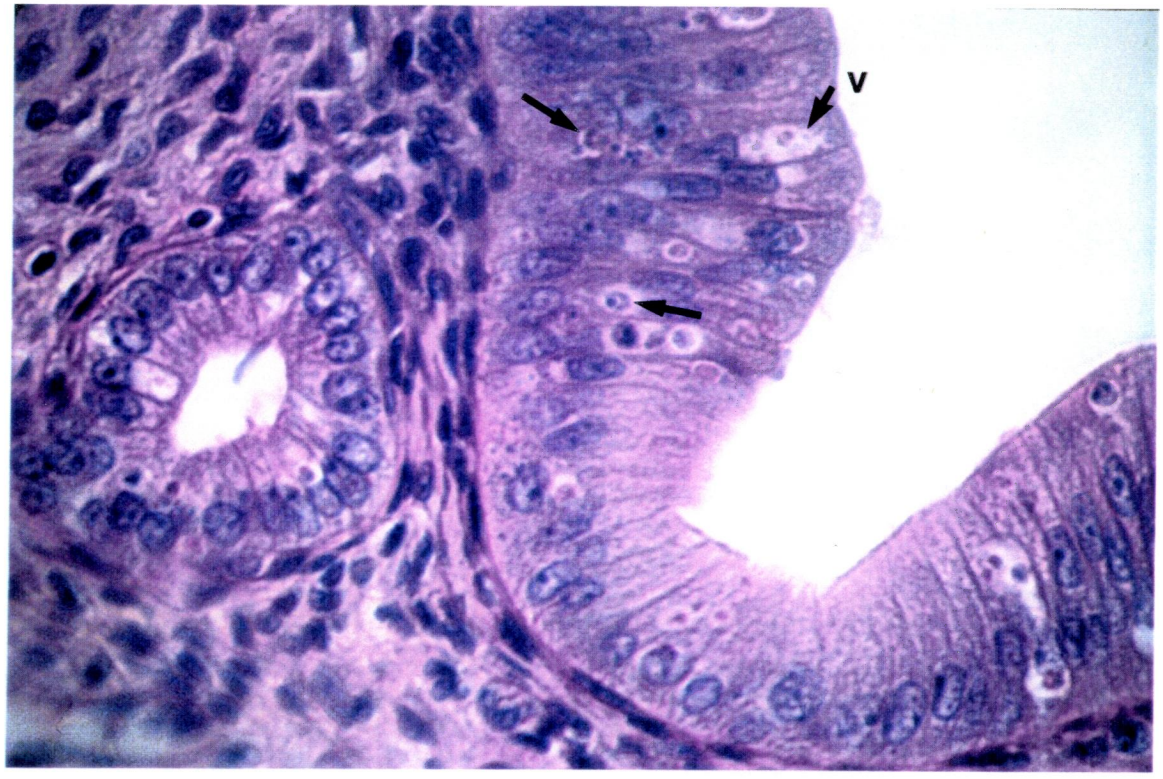


Figure 21. 30 day immature rat, 48 h post 40 IU PMSG, 2472X.

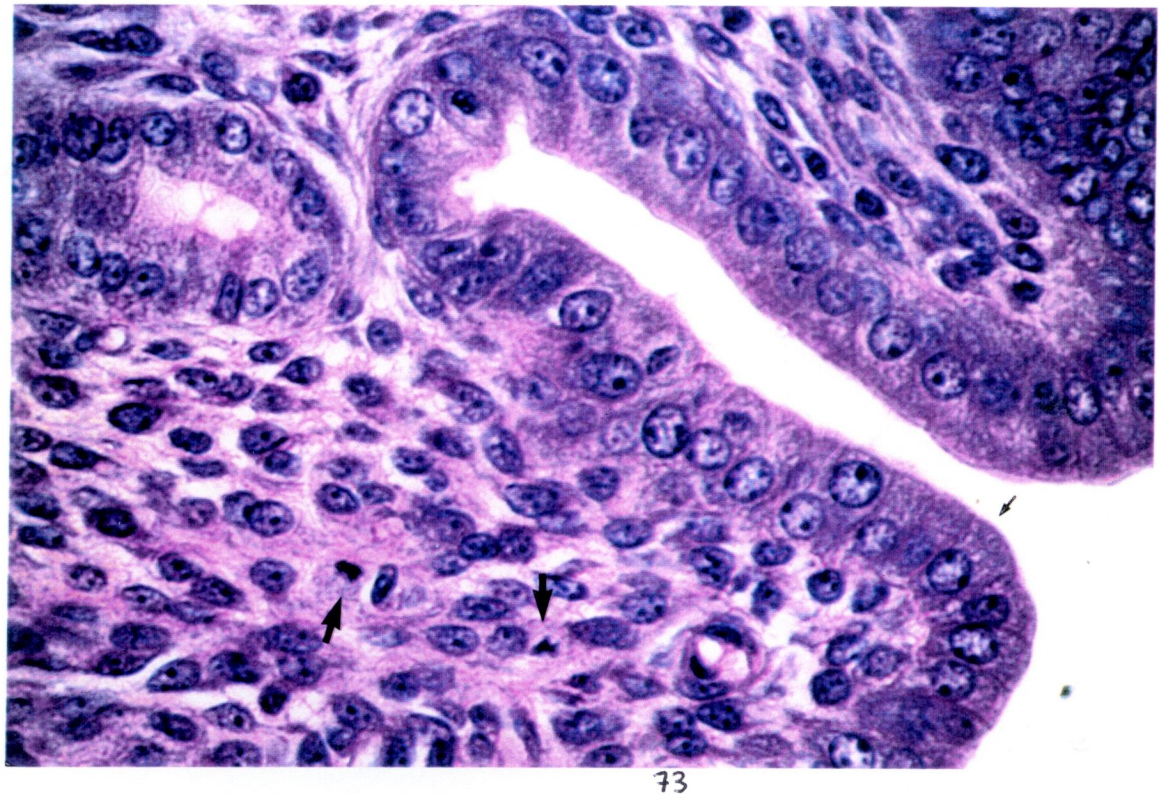


Figure 22. 35 day immature rat, 168 h post 4 IU PMSG, 153X. Note that the open uterine lumen (L) has several simple infolds. There are a few more glands (arrows) which are predominantly in the antimesometrial end (am). If mating or fertilization had occurred, this would be the time of implantation. The serum steroid concentrations in this rat were estradiol 134 pg/mL, progesterone 89 ng/mL and androgens 0.84 ng/mL.

Figure 23. 35 day immature rat, 168 h post 40 IU PMSG, 153X. Note the numerous invaginations into the endometrium. The luminal epithelial cells extend deeply into the subepithelial stroma, forming many flaccid projections with papillary extensions (arrows). Contrast the appearance of the lumen to the simpler lumen found in the mature rat (Figures 10, 12, 14 & 16) or in the 4 IU PMSG treated rat at 168 h (Figure 22). If fertilization had occurred, this would be the appearance of the uterus at the time of implantation. Note also, the increased size of the entire uterus (hypertrophy). The serum steroid concentrations in this rat were estradiol 153 pg/mL, progesterone 130 ng/mL and androgens 1.13 ng/mL.

Figure 22. 35 day immature rat, 168 h post 4 IU PMSG, 153X.

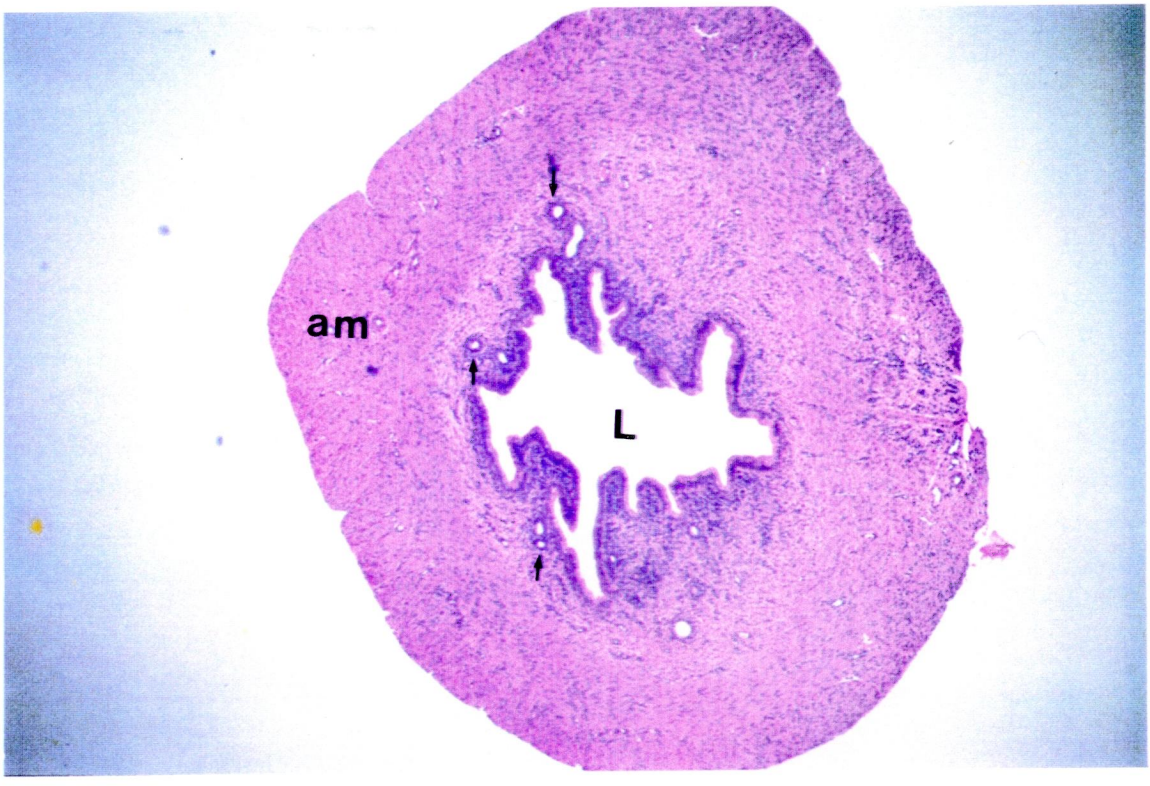


Figure 23. 35 day immature rat, 168 h post 40 IU PMSG, 153X.

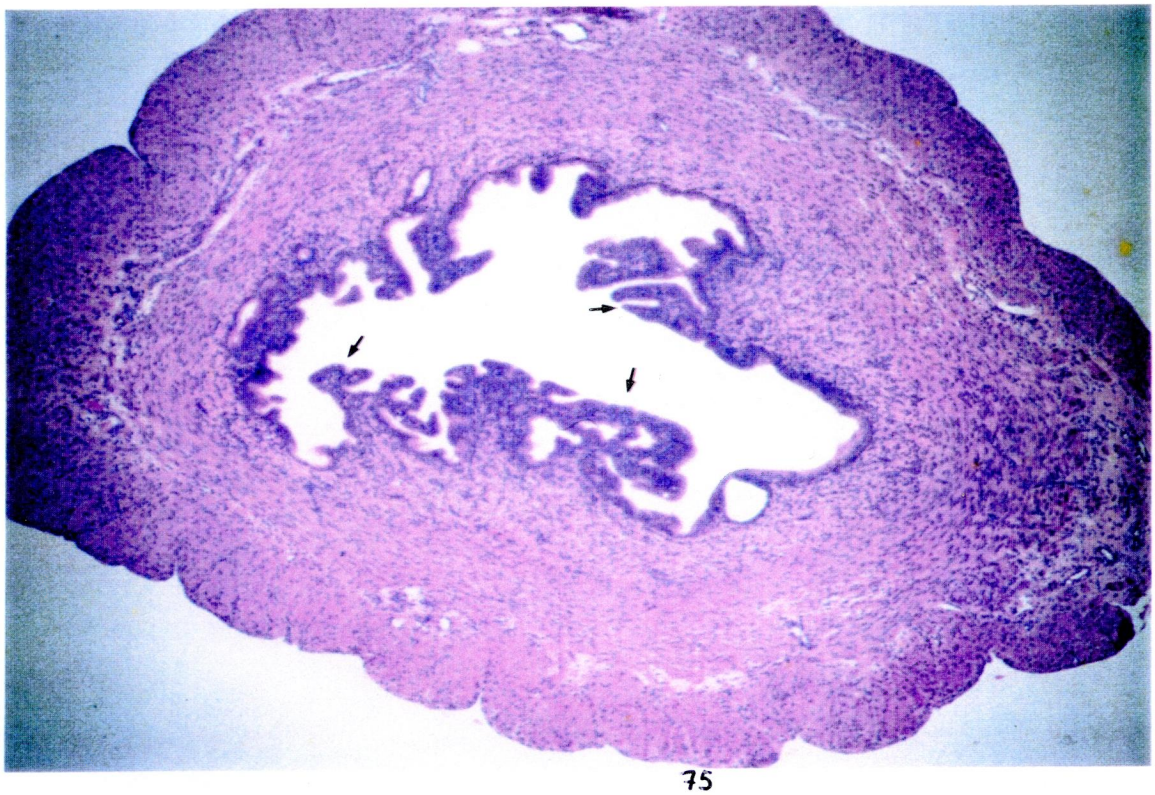


Figure 24. 35 day immature rat, 168 h post 4 IU PMSG, 2472X. Note the tall packed columnar surface epithelial cells, and the vacuoles (arrow) in many epithelial cells. There is also karyorrhectic debris present (small arrow) and notice the difference between the uterine luminal epithelium and the epithelial cells lining the gland (g). The serum steroid concentrations in this rat were estradiol 95 pg/mL, progesterone 8.5 ng/mL and androgens 0.4 ng/mL.

Figure 25. 35 day immature rat, 168 h post 40 IU PMSG, 2472X. Note the large amount of secretory material (arrowhead) as well as the scanty intervening stroma. No glands are identifiable.

Figure 24. 35 day immature rat, 168 h post 4 IU PMSG, 2472X.

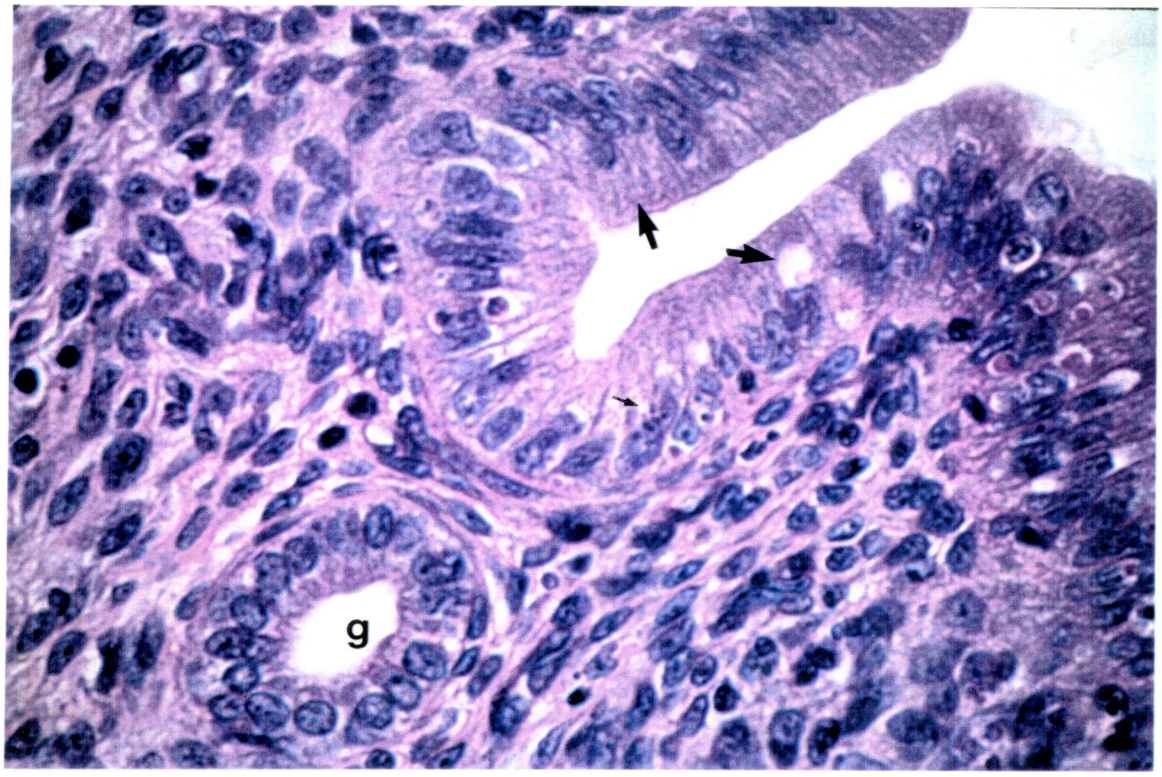
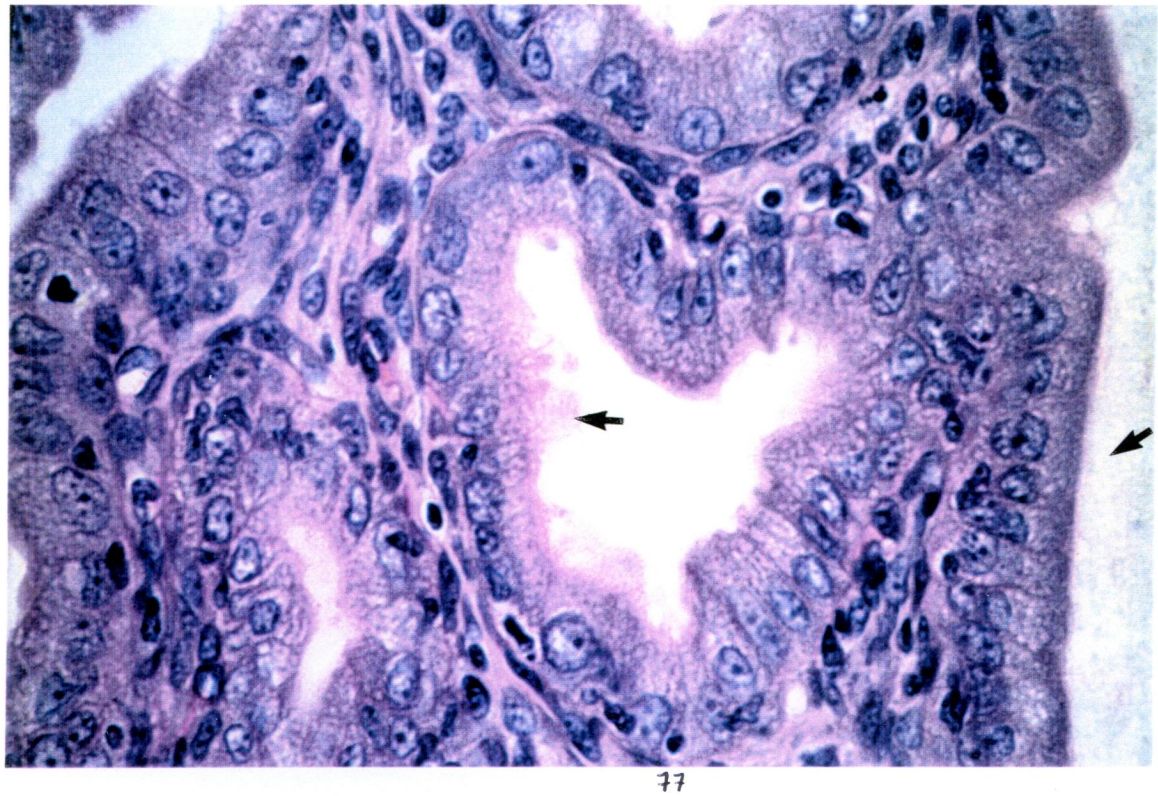


Figure 25. 35 day immature rat, 168 h post 40 IU PMSG, 2472X.



Figures 26 - 27 are photographs of Bouin fixed uteri before paraplast processing, from Mature Rats and 4 IU and 40 IU PMSG treated rats, 168 h post PMSG treatment.

Figure 26. Mature rat uteri during proestrus, estrus, metestrus and diestrus. Note duplex uteri.

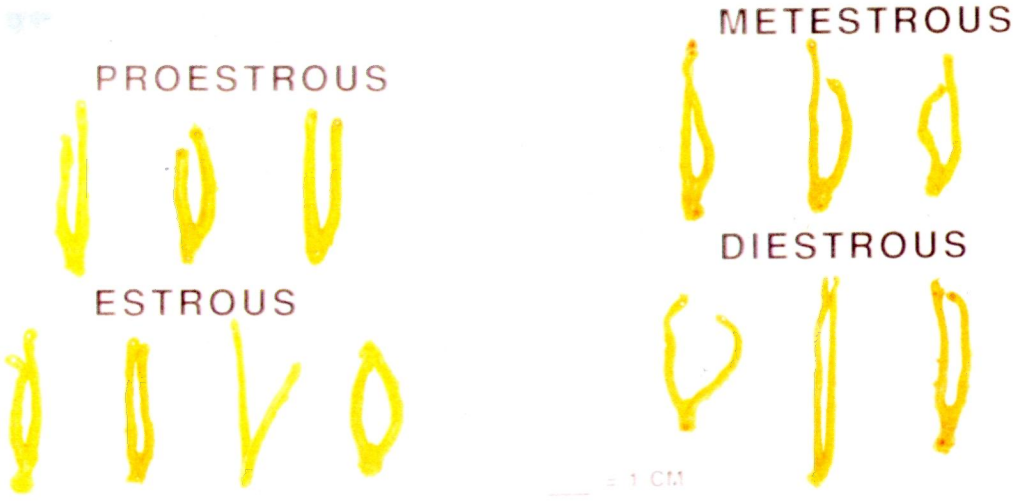
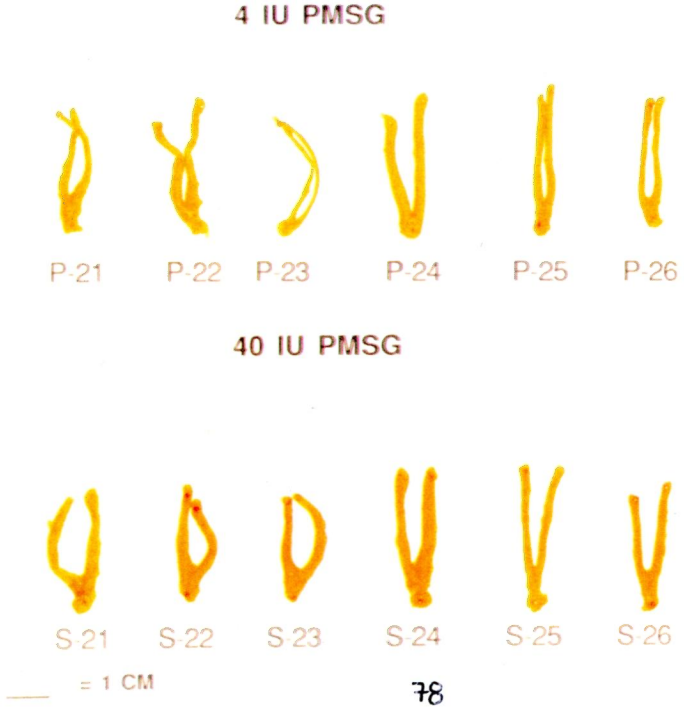


Figure 27. Immature rat uteri, 168 h after treatment with a 4 IU PMSG and a 40 IU PMSG dose.



3.5.2 Uterine luminal perimeters

The results from the measurement of uterine luminal perimeters are presented as a ratio of the inner uterine luminal perimeters:outer uterine perimeter (lumenP:lumenO) for the untreated mature rats and the 4 IU and 40 IU PMSG treated immature rats are shown in Figure 28 a & b.

In mature rats, the ratio of the perimeter lengths during proestrus (mean 0.67 ± 0.07 mm) and estrus (mean 0.71 ± 0.11 mm) were not different from each other but were longer than uterine luminal perimeters found at metestrus (mean 0.41 ± 0.02 mm) and diestrus (mean 0.27 ± 0.03 mm) ($p<0.05$).

The ratio of the uterine luminal perimeter lengths in 4 IU PMSG treated rats displayed a cyclic pattern. The luminal perimeters ranged from a mean of 0.84 ± 0.04 mm at 48 h to a mean of 0.3 ± 0.07 mm at 216 h post 4 IU PMSG injection ($p<0.05$). The ratio of uterine luminal perimeter lengths in 40 IU PMSG treated rats, did not display a cyclic pattern but rather steadily increased in length during the course of the experiment. In the 40 IU PMSG treated rats, the ratio of the uterine luminal length ranged from a mean of 0.55 ± 0.16 mm at 24 h and a mean of 1.03 ± 0.09 mm at 168 h ($p<0.05$). The ratio of the lumen perimeters were similar between the 4 IU and 40 IU PMSG treated groups during the "pre" stage, but the ratio was higher in the 40 IU PMSG group during the "post" stage ($p<0.05$).

Figure 28a. Ratio of the inner uterine luminal perimeter to the outer uterine perimeter in mature rats

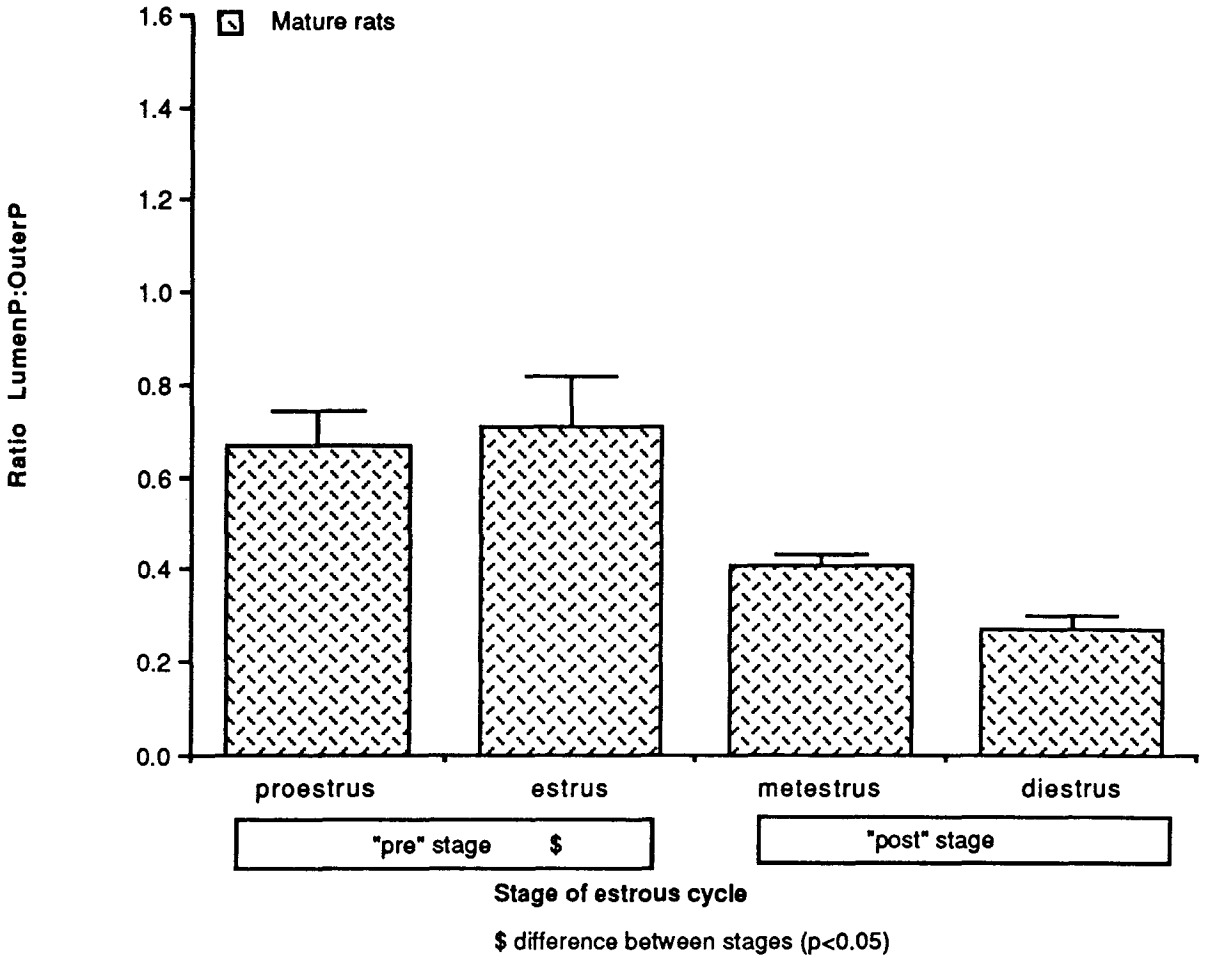
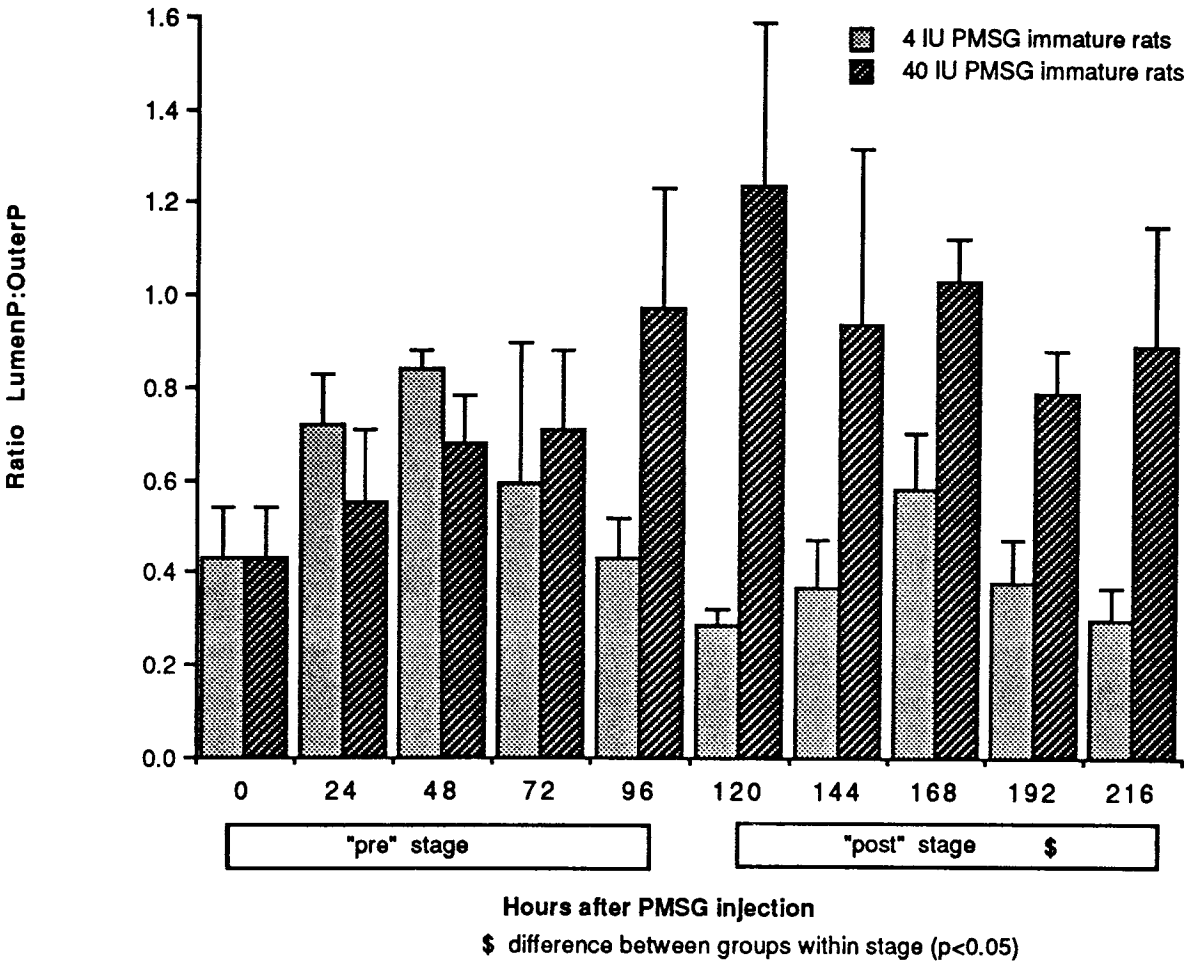


Figure 28b. Ratio of the inner uterine luminal perimeter to the outer uterine perimeter in 4 IU & 40 IU PMSG treated immature rats



3.5.3 Mitotic figures

The results of the number of mitotic figures counted in the uterine luminal epithelium and subepithelial stroma for the untreated mature rats and the 4 IU and 40 IU PMSG treated immature rats are shown in Figures 29 a, b & c.

3.5.3.1 Mitotic figures in the luminal epithelium

In the mature rat, the number of mitotic figures counted in the luminal epithelium varied (4.7 ± 3.2 in proestrus, 2.75 ± 2.6 in estrus, 2.3 ± 3.2 in metestrus and 6.7 ± 3.5 in diestrus). The number of epithelial mitotic figures in the 4 IU PMSG treated rats ranged from a mean of 0 at 48 and 144 h to a mean 13.0 ± 15.4 at 192 h. The number of epithelial mitotic figures counted in the 40 IU PMSG treated rats ranged from a mean of 0 at 192 h to a mean of 13.5 ± 0.71 at 48 h. The number of mitotic figures counted in the luminal epithelium were similar in the 40 IU PMSG, 4 IU PMSG treated immature rats and the untreated mature rats.

3.5.3.2 Mitotic figures in the subepithelial stroma

The number of mitotic figures counted in the subepithelial stroma in untreated mature rats varied (2.33 ± 3.21 in proestrus, 1.75 ± 1.71 in estrus, 0.33 ± 0.58 in metestrus and 0 in diestrus). The number of mitotic figures counted in the 4 IU PMSG treated group ranged from a mean of 0 at 216 h to a mean 17.7 ± 10.7 at 24 h. The number of stromal mitotic figures in the 40 IU PMSG treated group, ranged from a mean of 0 at 192 and 216 h to a mean of 21.7 ± 9.6 at 24 h ($p<0.05$). During the "post" stage the number of stromal mitotic figures in the 40 IU PMSG treated rats was decreased when compared to the 4 IU PMSG treated immature rats ($p<0.05$).

Figure 29a. Number of epithelial and stromal mitotic figures in mature rats

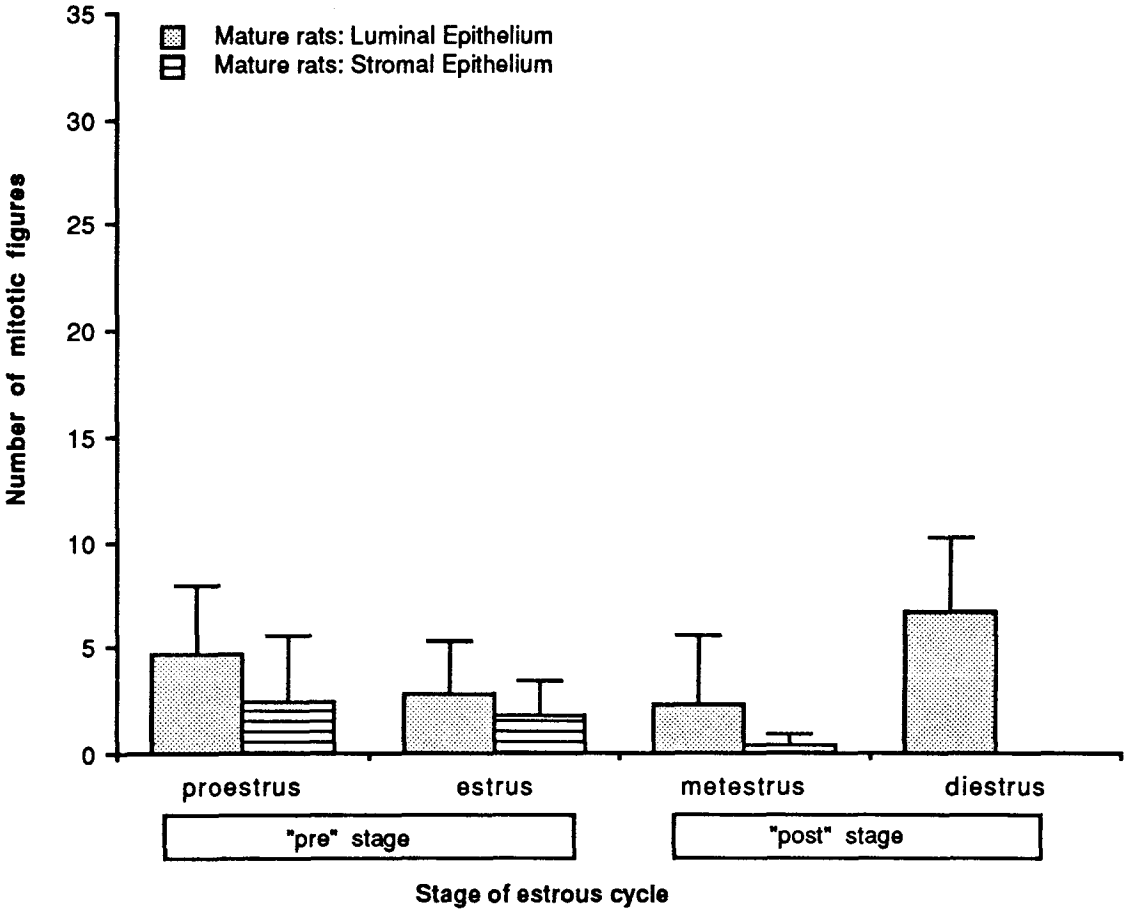


Figure 29b. Number of epithelial mitotic figures in 4 IU and 40 IU PMSG treated immature rats

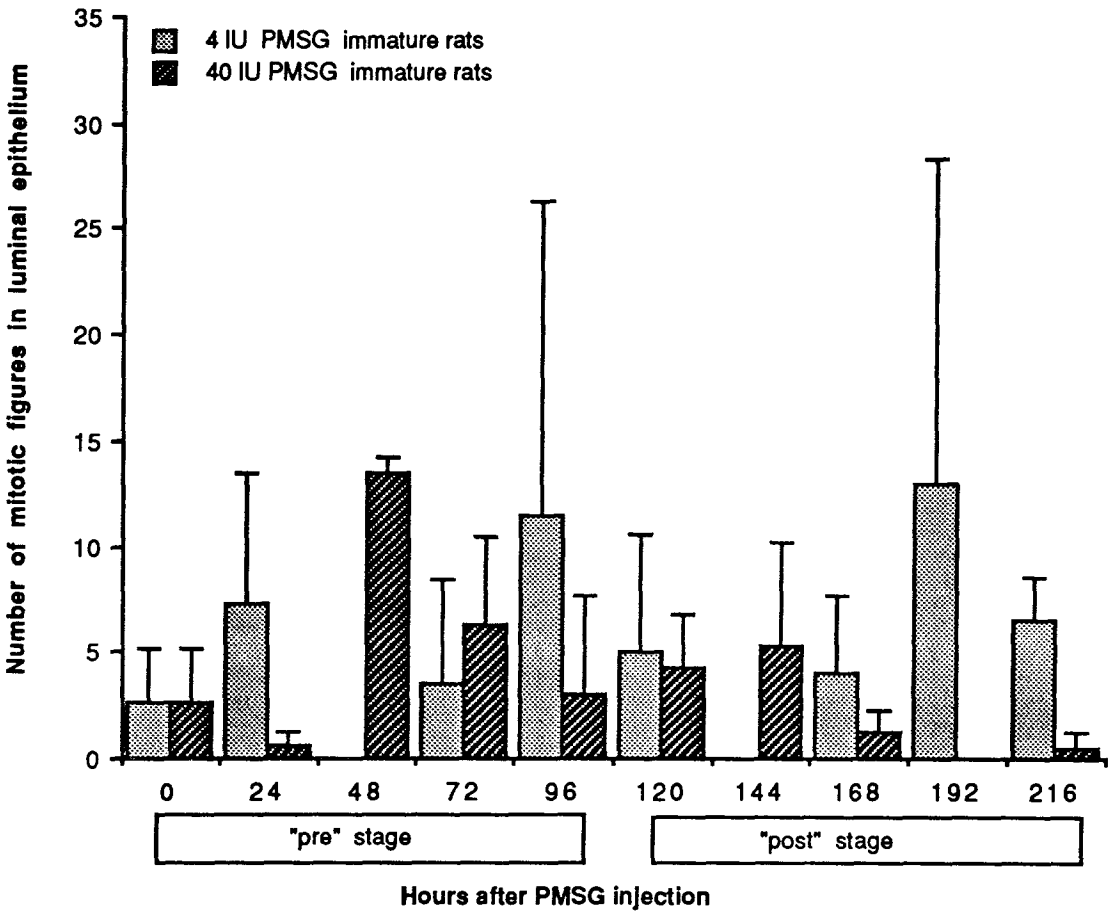
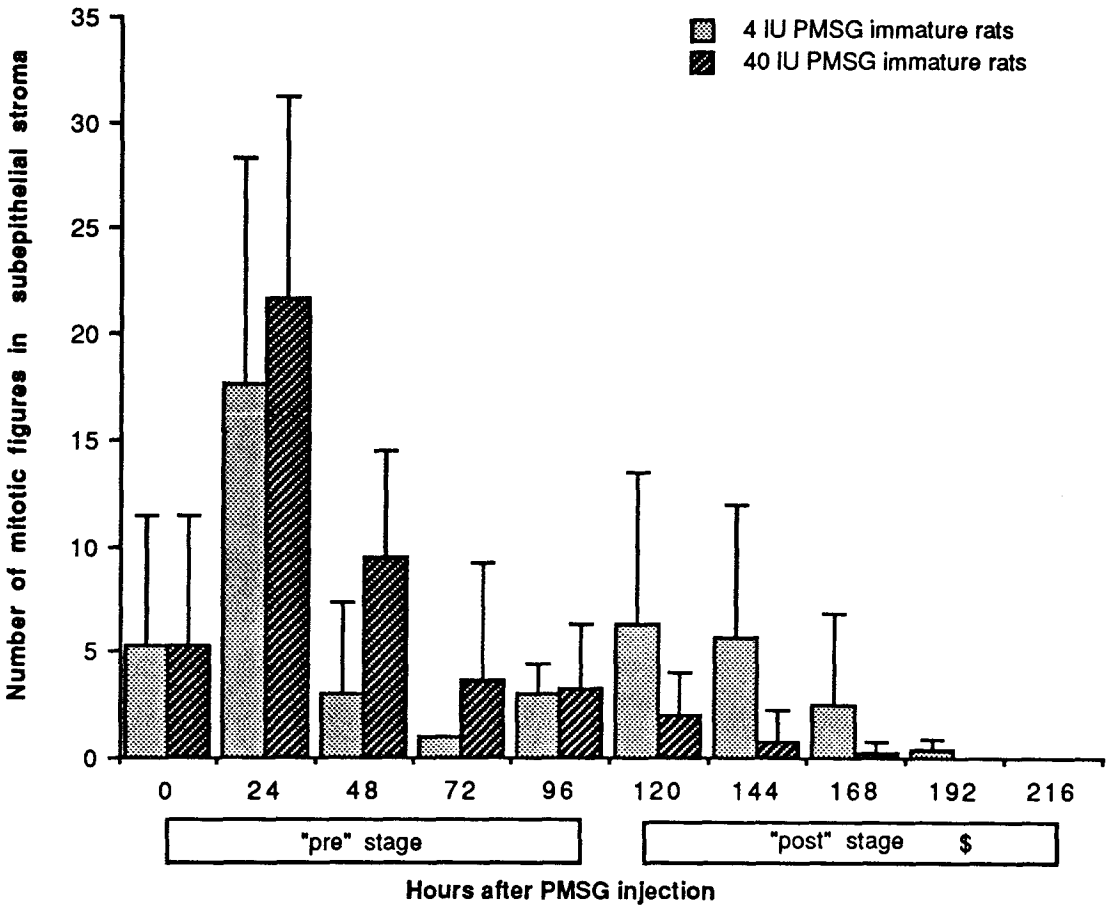


Figure 29c. Number of stromal mitotic figures in 4 IU and 40 IU PMSG treated immature rats



$ difference between groups within stage

3.6 Carbohydrate histochemistry

The results of the staining with the various carbohydrate histochemical methods from studies of the middle uterine horn are presented in Tables 8a through 9f. A comparison of the amount and intensity of carbohydrate staining of the glycocalyx, secretions and glands is presented in Tables 10a through 10c & 11, while a summary of the relative amount and composition of the glycocalyx and secretions is shown in Tables 12a through 12e. Photomicrographs from mature rat, 4 IU PMSG and 40 IU PMSG treated immature rat uteri, stained with the combination carbohydrate methods are shown in Appendix one (slides 1 - 24).

3.6.1 Glycocalyx

3.6.1.1 Amount of the glycocalyx

As shown in Table 10a, the amount of the glycocalyx in the oviductal, middle, cervical and the cervix-vagina regions in the 4 IU, 40 IU PMSG treated immature rats and untreated mature rats differed ($p<0.05$). Multiple comparisons indicated that the amount of the glycocalyx was increased in the 40 IU PMSG treated immature rats as compared to the 4 IU PMSG treated immature rats or the untreated mature rats ($p<0.001$). The ratio of sulphate:neutral sugars in the glycocalyx of the 40 IU PMSG treated rats was increased in the "post" stage ($p<0.001$) (Table 8b).

3.6.1.2 The intensity carbohydrate staining of the glycocalyx

The glycocalyx of the 4 IU PMSG, 40 IU PMSG treated immature and untreated mature rats differed in respect to the intensity of staining for sialic acid, sulphate ester and neutral sugars in the oviductal, middle, cervical, and cervix-vaginal regions (Table 8d) ($p<0.05$).

Multiple comparisons indicated an increase in neutral sugars in the 40 IU PMSG treated rats when compared either of the other animals ($p<0.001$). The neutral sugars in the glycocalyx in the 40 IU PMSG treated group were also increased in the "post" stage (Table 11) ($p<0.001$).

The ratio of sialic acid:neutral sugars (Table 8a) and the ratio of sulphate esters:neutral sugars (Table 8b) in the glycocalyx differed when the 4 IU PMSG, 40 IU PMSG immature rats and the mature rats were compared ($p<0.05$). However, multiple comparisons were not able to distinguish in which groups these changes occurred.

The ratio of sialic acid:neutral sugars in the glycocalyx of the 40 IU PMSG treated rats differed when the "pre" stage and "post" stages in the 4 IU PMSG treated rats and the untreated mature rats were compared (Table 11) ($p<0.001$). The change in the ratio of sialic acid:neutral sugars in the glycocalyx in the "post" stage of 40 IU PMSG treated immature rats appeared to be due to an increase in sialic acid. However, it was not possible to determine whether the amount of neutral sugars within the glycocalyx in the 40 IU PMSG immature rats "post" stage were unchanged, decreased or had a smaller increase when compared to sialic acid.

3.6.2 Free secretions in the uterine lumen

3.6.2.1 Amount of uterine secretions

As shown in Table 10b. the amount of the free secretions present in the oviductal, middle, cervical and cervix-vagina regions in the 40 IU PMSG, 4 IU PMSG treated immature rats and the untreated mature rats differed ($p<0.05$). Multiple comparisons indicated that the amount of free secretions were increased in the 40 IU PMSG treated rats ($p<0.001$). The amount of secretions in the 40 IU PMSG treated rats were increased in the "post" stage (Table 11).

3.6.2.2 The intensity carbohydrate staining of the uterine secretions

The uterine secretions in the 40 IU PMSG treated rats, 4 IU PMSG treated immature and the mature rats differed in respect to the intensity of staining for sialic acid, sulphate esters and neutral sugars in the free secretions in the oviductal, middle, cervical region and cervix-vaginal regions of the uteri (Table 8e) ($p<0.05$). Multiple comparisons indicated that there was an increase in sialic acid and neutral sugars in the secretions from 40 IU PMSG treated rats when compared to the 4 IU PMSG treated rats in the oviductal and middle regions of the uterine horn (Table 10b). Sialic acid was increased in the 40 IU PMSG treated rats in the middle and cervical regions of the uterine horn as compared to the mature rats ($p<0.001$). Sialic acid in the middle, cervical and the cervix vaginal regions of the uteri in the 40 IU PMSG treated rats was increased in the "post" stage (Table 11) ($p<0.001$).

The ratios of sialic acid:neutral sugars and sulphate:neutral sugars (Table 10e) of the uterine secretions in the oviductal, middle and cervical regions of the uteri differed in the 4 IU PMSG, 40 IU PMSG and the untreated mature rats ($p<0.05$). However, multiple comparisons were not able to distinguish in which groups these changes occurred.

3.6.3 Glands

3.6.3.1 Amount of glandular sulphate esters, neutral sugars and sialic acid

The glands of the 4 IU PMSG, 40 IU PMSG treated immature and the untreated mature rats differed in the amount of sulphate and neutral sugars present in the middle and cervical regions and differed in the amount of sialic acid in the oviductal region of the uterine horn (Table 9a) ($p<0.05$). However, multiple comparisons were not able to distinguish in which group these differences occurred.

3.6.3.2 The intensity carbohydrate staining in the glands

The glands of the 4 IU PMSG, 40 IU PMSG treated immature rats and the untreated mature rat differed with respect to the intensity of staining for sulphate (Table 8d), the ratio of sialic acid:sulphate esters (Table 8c) and the ratio neutral sugars:sulphate esters (Table 8b). The differences in the ratios was likely due to an increase in the presence of sulphate esters. However, it was not possible to determine whether there were no change, a decrease or smaller increase in the amount of neutral sugars or sialic acid present as compared to the sulphate esters.

3.6.4 Summary of carbohydrate staining results

The amount of the epithelial glycocalyx and uterine secretions were similar in the 4 IU PMSG treated immature rats and the untreated mature rats. However, the amount of the glycocalyx and uterine secretions were increased in the 40 IU PMSG treated immature rats (Table 12a).

The glycocalyx in both the groups of immature rats and the untreated mature rats contained 0-sulphate esters, neutral sugars and sialic acid. However, the glycocalyx in the 40 IU PMSG treated immature rats, had an increase in neutral sugars present when compared to the 4 IU PMSG treated or mature rats (Table 12c).

The uterine secretions in both the groups of immature rats and the untreated mature rats contained 0-sulphate esters, neutral sugars and sialic acid. However, the uterine secretions in the 40 IU PMSG treated immature rats, had an increase in neutral sugars and sialic acid present when compared to the 4 IU PMSG treated or mature rats (Table 12b).

The glycocalyx in the 40 IU PMSG treated immature rats had an increase in neutral sugars in the "post" stage (Table 12e). The uterine secretions in the 40 IU PMSG treated

immature rats had increased sialic acid in the "post" stage (Table 12d).

3.6.5 Subepithelial stroma (sulphate esters)

Sulphate esters in the subepithelial stroma from the 4 IU PMSG, 40 IU PMSG treated immature rats and the untreated mature rats in the oviductal, middle, cervical and the cervix vaginal region differed ($p<0.05$). Multiple comparisons revealed that these differences occurred between the 40 IU PMSG treated rats and the untreated mature rats ($p<0.001$). In the oviductal region, and there were also differences within the 4 IU PMSG treated group, when the "pre" stage versus "post" stages were compared ($p<0.001$).

Table 8a. Results of the ratio of sialic acids:neutral sugars, using the PA*/T/Bh/PAS/KOH method, in the glycocalyx, uterine secretions and glands, from the middle uterine cross-section, in 4 IU PMSG, 40 IU PMSG treated immature rats and untreated mature rats

Hrs Post PMSG	Glycocalyx 4 IU *	Glycocalyx 40 IU * $	Uterine Secretions 4 IU *	Uterine Secretions 40 IU * $	Glands 4 IU	Glands 40 IU
0	6, 6, 6		np, np, np		np,np, np	
24	4, 6, 6	4, 6, 6	np, np, 6	np, np, 4	np, np, np	np, np, 4
48	5, 6	4, 6, 6	5, 6	4, 6, 6	np, 4	np,np, np
72	4, 6, 6	4, 5, 6	np, 4, 6	4, 4, 6	4, 4, 4	4, 4, 4
96	4, 4	4, 4, 4, 4	np, 4	4, 4, 4, 5	4, 4	np, 4 np, 4,
120	4, 4, 4	6, 6, 6	np, np, np	6, 6, 6	3, 4, np	?, 4, np
144	4, 5, 5, 6	4, 4, 4, 4	np,np,np, 5	3, 3, 4, 6	np,3,3.5	np, np np, np
168	4, 4, 4	3, 3, 3, 3	3, 4, np	3, 3, 3, 3	np, np, 4	np, np ?, ?
192	4, 4, 4, 3	3, 3, 3	np,np,np, 3	np, 3, 3	np,np,np, np	?, ?, ?
216	4, 4, np	3, 4, 2	np, np,np	2, 3, 4	4, 4,np	?, ?, ?

Stage of Cycle	Glycocalyx *	Uterine Secretions *	Glands
Proestrus	6, 6, 6	np, 6, 6	np, np, np
Estrus	6, 6, 4, 6	6, np, 6, np	6, 6, 7, 7
Metestrus	4, 4, 4	6, 4, 5	6, 4, np
Diestrus	4, 4, 4	np, np, np	np, 4, 4

Key: np= not present 1 = blue (sialic acid) 2 = blue-purple 3 = purple-blue
4 = purple 5 = purple-red 6 = red-purple
7 = red (neutral sugars) ? = glands not distinguishable
Each number separated by a coma (,) represents results from one rat
Glycocalyx = apical surface of luminal epithelium; Secretions = free material in the uterine lumen; Glands = the content and/or lining of the glands
* difference in the ratio of sialic acid:neutral sugars; 4 IU PMSG versus 40 IU PMSG versus untreated mature rats ($p<0.05$)
$ difference within 40 IU PMSG Group pre versus post stage ($p<0.001$)

Table 8b. Results of staining for the ratio of sulphate esters:neutral sugars, using the PA*/Bh/AB1.0/PAS method, in the glycocalyx, uterine secretions and glands, from the middle uterine cross-section, in 4 IU PMSG, 40 IU PMSG treated immature rats and untreated mature rats

Hours Post PMSG	Glycocalyx 4 IU *	Glycocalyx 40 IU *	Uterine Secretions 4 IU *	Uterine Secretions 40 IU *	Glands 4 IU	Glands 40 IU
0	4, 4		4, 4		np, 4	
24	3, 3, 3	3, 3, 5	np, np, np	np, np, 3	np, np, 3	np, np, 3
48	3, 3	3, 3, 3	3, 3	3, 3, 6	8, 8	6, np, np
72	3, 3,3	3, 3, 4	3, 4, 5	3, 3, 3	6, 8, 8	3, 3, 6
96	3, 4	3, 3, 5	np, 7	3, 3, 4	7, 3	np, 5, 6
120	4, 3,3	3, 3, 3	4, np, np	3, 3, (3, 4)	3, 6, 6	8, ?, ?
144	3, 3, 3, 5	3, 3, 3, 5	5, np, np, np,	5, 3, 3, 4	7, 7, np, 6	?, ?, ?, ?
168	3, 3, 3, 4	3, 3, 3, 3	3, 3, 4, np	3.5, 3, 3, 3	5, 6, np, np	7, ?, ?, ?
192	3, 3, 4	4, 6, 6	np, np, 3.5	5.5, 6, (6,4)	3, 3, 3	?, ?, ?
216	3, 3, np	5, 5, 5	3, np, np	7, 7, 6	3, 3, np	?, ?, ?

Stage of Cycle	Glycocalyx *	Uterine Secretions *	Glands
Proestrus	3, 3, 3	3, 4, np	np, np, np
Estrus	3, 3, 3, 3	5, 3, 3, np	7, 7, 8, np
Metestrus	3, 3, 3	5, 7, 7	3, 4, 7
Diestrus	4, 4, 3	np, np, 3	3, 3, 3

Key: np= not present 1 = aquamarine (sulphate esters) 2 = blue
3 = blue-purple 4 = purple-blue 5 = purple
6 = purple-red 7 = red-purple 8 = red (neutral sugars)
? = glands not distinguishable
Each number separated by a coma (,) represents results from one rat
The 0 hour rats were not injected with PMSG
Glycocalyx = apical surface of luminal epithelium
Secretions = free material in the uterine lumen
Glands = the content and/or lining of the glands
* difference in the ratio sulphate:neutral sugars; 4 IU PMSG versus 40 IU PMSG versus untreated mature rats ($p<0.05$)

Table 8c. Results of staining for the ratio of sulphate esters:sialic acid, using the PA*/AB1.0/S method, in the glycocalyx, uterine secretions and glands, from the middle uterine cross-section, in 4 IU PMSG, 40 IU PMSG treated immature rats and untreated mature rats

Hrs	Glycocalyx 4 IU	Glycocalyx 40 IU	Uterine Secretions 4 IU	Uterine Secretions 40 IU	Glands 4 IU	Glands 40 IU
0	3, 4		3, 4		np, np	
24	3, 3, 3	3, 3	3, 3, 3	3, np	np,np,np	3
48	3, 3	3, 3, 3	3, 3	np, 3, 3	np, 3, 6	np,7, np
72	3, 3, 4	2, 3, 3	3, 4.5, np	2, 3, 3	3, 6, np	3,np, np
96	3, 4	1, 2, 2	np, 4	1, 2, 2	5.5, 4	?, ?, 8
120	2, 3, 3, 3	2, 2, 2	2, 3, 3, np	2, 2, 5	7,4,3, np	?, ?, 6
144	3, 3, 4	2, 2, 4	np,np, np	4, 2, 5	5.5, 6,np	?, ?, ?
168	2, 2, 3, 3	2, 2, 2, 2	5, 3, 2, np	2, 2, 2, 2	3,3,np,np	?,?, ?, 7
192	3, 3, np	3, 5	3, np, np	5, (4, 6)	3, 3, np	np, ?
216	3, 3, np	3, 4, 5	3, np, np	4, 6, 5	3, 3, np	6, ?, ?

Stage of cycle	Glycocalyx	Uterine Secretions	Glands
Proestrus	3, 3	4, np	np, np
Estrus	3, 3, 3	4, np, 4	9, 9, (5, 9)
Metestrus	4, 4	5, 5	np, (9, 3)
Diestrus	4, (3, 4)	np, np	np, 4

np= not present
1 = aquamarine (sulphate esters) 2 = blue-aquamarine
3 = blue 4 = blue-purple
5 = purple-blue 6 = purple
7 = purple-red 8 = red-purple
9 = red (sialic acid) ? = glands not distinguishable

Each number separated by a coma (,) represents results from one rat
The 0 hour rats were not injected with PMSG

Glycocalyx = apical surface of luminal epithelium
Secretions = free material in the uterine lumen
Glands = the content and/or lining of the glands

No differences in the ratio of sulphate esters:sialic acid

Table 8d. Results for staining "intensity" (density of colour) of the glycocalyx for sulphate esters, sialic acid and neutral sugars as stained by the AB1.0, PA*S and PA*/Bh/PAS methods, in the middle uterine horn cross-section

	4 IU*	40 IU*	4 IU*	40 IU*	4 IU *#	40 IU $^#*
H	AB1.0	AB1.0	PA*S	PA*S	N.S.	N.S.
0	.5, 1.5, .5		.5, .5, .5		1, 1.5, .5	
24	1.5, 2, 2.5	1, .5, 3	1, 1, 1.5	.5, .5, .5	1, 1, 2	1, 1, 2
48	3, 3	2.5, 2.5, 3	.5, 2	.5, .5, .5	2, 2,	1, 2.5
72	3, 3.5, 2.5	3, 3, 2	2, 2, 1.5	1.5, 1.5, 1.5	1.5, 2, 1	1.5,2
96	2, 4	3, 3, 4, 2	1, 1.5	1, 1.5, 1.5, .5	1, 2	2, 2, 2, 2
120	2, 2.5, 2	4, 4, 4	0, 1.5, 1.5	2, 2.5, 1.5	1, 1, 1	3, 3.5, 3.5
144	1, 2, 2, 3	3.5, 4, 3.5, 3.5	0, .5, .5, .5	2, 2, 2, 2.5	1.5, 1, 1.5, 1	3, 3.5, 3, 3
168	3, 2, 3, 2.5	4, 4, 4, 4	2, .5, 0, 0	2, 2.5, 2.5, 2.5	2, 1.5, 2, 1.5	3, 2.5, 2.5, 3
192	2, 0, 2.5	1.5, 3.5	1.5, 0, .5	1.5, 2.5	.5, 0, 1.5	3, 2, 3
216	0, 2, 2.5	3, 4, 3	0, 1, 0	2, 2, 2	1, 1, 0	3, 3, 3

KEY: np = not present 0 = no staining 0.5 = scanty (+/-)
1 = trace 2 = some
3 = moderate 4 = maximal staining

Each number separated by a coma (,) represents results from one rat
The 0 hour rats were not injected with PMSG
Glycocalyx = apical surface of luminal epithelium
Secretions = free material in the uterine lumen
Glands = the content and/or lining of the glands

* difference 4 IU PMSG versus 40 IU PMSG versus untreated mature rats ($p<0.05$)
# difference 4 IU PMSG versus 40 IU PMSG treated rats ($p<0.001$)
$ difference within 40 IU PMSG group; pre versus post stage ($p<0.001$)
^ difference 40 IU PMSG treated immature rats versus untreated mature rats ($p<0.001$)

Table 8e. Results for staining "intensity" (density of colour) of the uterine secretions for sulphate esters, sialic acid and neutral sugars as stained by the AB1.0, PA*S and PA*/Bh/PAS Methods in the middle uterine horn cross-section

	Secretions					
	4 IU	40 IU	4 IU	40 IU	4 IU	40 IU
Hrs	AB1.0 *	AB1.0 *	PA*S * #	PA*S *#^$	N.S. * #	N.S. *#^
0	1,1.5,.5		.5,1.5, .5		1,1.5,.5	
24	2.5,1.5,3.5	2,np,3	2,1,2	1,1,0	2,1,2.5	np,1,2.5
48	3,3.5	2.5,2.5,3.5	2.5,.5	1,1.5,1	2,3	2,2.5,2
72	3,4,2.5	2.5,4,2	1,2.5,2	1.5,2.5,1.5	1.5,3.5,1	1,2
96	2,4	2,3.5,4,3	1,2	2,3,1.5,.5	1,2.5	2,2,4,4
120	2,2.5,3	4,4,4	0,1.5,np	3,3.5,3	1,2,2 pat	4,4,4
144	np,3,np,3	3.5,4,4,4	.5,1,1.5 pat,np	2.5,3,3.5,3.5	1.5,2,2.5,1.5 pat	3.5,4,4,4
168	3,2,4, 2.5	4,4,4,4	3,.5,.5,3 pat	3,3.5, 3.5,3	3,2,3.5,1.5	4,3.5,4,3.5
192	2,0,3	1.5,4	.5,0,.5	2,3.5 pat	.5,0,1.5	4,2
216	0,2.5,2	4,4,3	0,.5,0	4,4,2.5	0,np,np	4,4,3

KEY: np = not present 0 = no staining 0.5 = scanty (+/-)
1 = trace 2 = some
3 = moderate 4 = maximal staining

Each number separated by a coma (,) represents results from one rat
The 0 hour rats were not injected with PMSG
Glycocalyx = apical surface of luminal epithelium
Secretions = free material in the uterine lumen
Glands = the content and/or lining of the glands

* difference 4 IU PMSG versus 40 IU PMSG versus untreated mature rats ($p<0.05$)
# difference 4 IU versus 40 IU PMSG treated rats ($p<0.001$)
$ difference within 40 IU PMSG Group pre versus post stage ($p<0.001$)
^ difference 40 IU PMSG Group versus untreated mature rats ($p<0.001$)

Table 9a. Results for the "amount" (relative area) for the ratio of neutral sugars:sialic acid as stained by the PA*/T/Bh/PAS/KOH method in the glycocalyx, uterine secretions, and glands from the middle uterine horn cross-section, in 4 IU PMSG, 40 IU PMSG treated immature rats and untreated mature rats

Hrs Post PMSG	Glycocalyx 4 IU *	Glycocalyx 40 IU * #	Uterine Secretions 4 IU *# ^$	Uterine Secretions 40 IU *#^$	Glands 4 IU	Glands 40 IU
0	1, .5, 1		np,np, np		np,np,np	
24	2, 1.5, 2	.5, .5, 2	np, np, 2	np,np,2	np,np,np	np, np, 1
48	2, 2	1, 1, 1.5	2, 1	.5, 1	np, .5	np,np,np
72	2, 2, 1	1.5, 1.5, 1.5	np, 3, 1	1, 1.5, 2	.5, 1, 1.5	1.5, .5, 1
96	1, 2	2, 2, 2, 2	np, 1	1.5,3,2.5,	1, 1	np,np,1,2
120	1, 1, 1	2, 2, 2	np, np,np	3, 3, 4	1, 1,np	?, 2, np
144	1, 1, 1, .5	2, 2, .5, 2	np, np, np, 1.5	2, 3, 3, 4	np, 1, 1, 1.5	np, np, np, np
168	2, 1, .5	2,2,2.5, 2.5	1, 2, np	3,3,3, 3.5	.5,np, np	np,np,? ?
192	1.5, 1.5, .5, 1	2, 2.5, 2.5	.5, np, np, np	np, 4, 2	np,np,np , np	?, ?, ?
216	.5, 1, np	3, 3, 2.5	np, np,np	3.5, 4, 3	1, 1,np	?, ?, ?

Stage of cycle	Glycocalyx *	Uterine Secretions * ^	Glands
Proestrus	0.5, 1.5, 1.5	np, 1.5, 1	np, np, np
Estrus	1.5, 1.5, 2, 2	1, np, 1, np	0.5 (np-3), (.5 - 2.5), (np - 3)
Metestrus	1.5, 1.5, 2.5	1, 1, 1	1, .5, np
Diestrus	1, .5, 1.5	np, np, np	np, 1, 1.5

KEY: np = not present 0 = no staining 0.5 = scanty (+/-) 1 = trace 2 = some
3 = moderate 4 = maximal staining
Each number separated by a coma (,) represents results from one rat
Glycocalyx = apical surface of luminal epithelium
Secretions = free material in the uterine lumen
Glands = the content and/or lining of the glands
* difference 4 IU PMSG versus 40 IU PMSG versus untreated mature rats ($p<0.05$)
# difference 4 IU versus 40 IU PMSG treated rats ($p<0.001$)
$ difference within 40 IU PMSG Group pre versus post ($p<0.001$)
^ difference 40 IU PMSG Group versus untreated mature rats ($p<0.001$)

Table 9b. Results for the "amount" (relative area) of the glycocalyx, uterine secretions and glands, from the middle uterine horn cross-section, as stained by the PA*/Bh/AB1.0/PAS method

Hours Post PMSG	Glycocalyx 4 IU * #	Glycocalyx 40 IU * # ^ $	Uterine Secretions 4 IU *	Uterine Secretions 40 IU *	Glands 4 IU	Glands 40 IU
0	1.5, 1.5		1.5, 1		np, .5	
24	2, 2, 2	1.5, 1, 2	np,np, np	np, np, 3	np,np,1	np,np, .5
48	2, 2	2, 2, 1.5	2, 1	2, 1, .5	.5, .5	.5,np, np
72	2.5, 2, 2	2, 2, 2	1, 3, 1	1.5, 2, 2.5	1, 1, 1	1.5,1,2
96	2, 2	2, 2, 2	1, np	3.5, 3, 3	1, 1	np, 1.5, 2
120	2,2,2	3.5, 3, 3	1, np, np	3, 3, 3	1.5, .5, .5	3, ?, ?
144	1, 2, 2, 2	3, 2.5, 3, 3	np, np, np, 1	3, 2, 3, 3.5	2, 1.5, 2, np	?, ?, ?, ?
168	2.5, 2, 2, 2	3, 3.5, 3.5, 3.5	1, 1, 2, np	4, 3.5, 3, 3.5	.5, np, np, 1.5	4, ?, ?, ?
192	2, 2, 2	2, 2.5, 2.5	1, np, np	1.5, 3, 2	.5, .5, .5	?, ?, ?
216	1.5,1.5,np	3.5, 3.5, 4	.5, np, np	4, 4, 3.5	.5,.5,np	?, ?, ?

Stage of Cycle	Glycocalyx * ^	Uterine Secretions *	Glands
Proestrus	2, 2, 2	2, 2, np	np, np, np
Estrus	2, 2, 2, 2	1, 1, 1, np	np, (np-1.5), 2, 1
Metestrus	2, 2, 2.5	.5, 1.5, 1.5	.5, .5, .5
Diestrus	2, 1.5, 1.5	np, np, 1.5	.5, .5, 1

KEY: np = not present 0 = no staining 0.5 = scanty (+/-)
1 = trace 2 = some
3 = moderate 4 = maximal staining

Each number separated by a coma (,) represents results from one rat
The 0 hour rats were not injected with PMSG
Glycocalyx = apical surface of luminal epithelium; Secretions = free material in the uterine lumen; Glands = the content and/or lining of the glands
* difference 4 IU PMSG versus 40 IU PMSG versus untreated mature rats ($p<0.05$)
# difference 4 IU versus 40 IU PMSG treated rats ($p<0.001$)
$ difference within 40 IU PMSG Group pre versus post ($p<0.001$)
^ difference 40 IU PMSG Group versus untreated mature rats ($p<0.001$)

Table 9c. Results for the "amount" (relative area) of the glycocalyx, uterine secretions and glands, from the middle uterine horn cross-section, as stained by the PA*/AB1.0/S method

Hours Post PMSG	Glycocalyx 4 IU *	Glycocalyx 40 IU*^ $#	Uterine Secretions 4 IU *	Uterine Secretions 40 IU *^$#	Glands 4 IU	Glands 40 IU
0	1.5, 1.5		1, 1		np, np	
24	2, 2, 1.5	1.5, 2	1, 2, 1.5	np, 2	np, np, np	1
48	2, 2	2.5, 2.5, 2.5	2, 2	np, 2.5, 2.5	np, 1, .5	np, 1.5, np
72	2, 2, 1.5	3, 2, 2	2, 2, np	2.5, 1, 2	1.5, 2, np	np, np, 1.5
96	1.5, 2.5	3, 3, 3	np, 3	2.5, 3, 2.5	2, 1.5	2, ?, ?
120	1, 2, 2, 2	3, 3.5, 3.5	.5, 1, np, 1	3, 3.5, 3	2, .5, .5, np	4, ?, ?
144	1.5, 2, 1.5	3.5, 3.5, 3.5	np, np, np	3, 3, 3.5	2.5, 1, np	?, ?, ?
168	3, 2.5, 2.5, 2	4, 4, 4, 4	1, 1, 1.5, np	4, 3.5, 3.5, 3	.5, .5, np, np	2, ?, ?, ?
192	1.5, 2, np	2.5, 3.5	1.5, np, np	1.5, 2	1.5, 1.5, np	np, ?
216	2, 1.5, np	3.5, 3.5, 3.5	1, np, np	4, 4, 4	1, 2, np	2, ?, ?

Stage of Cycle	Glycocalyx *	Uterine Secretions * ^	Glands
Proestrus	1.5, 2	1.5, np	np, np
Estrus	2, 2, 2	1,2, np	.5, 2, 1
Metestrus	2, 2	2.5, 1	np, 1
Diestrus	2, 2	np, np	np, 1

KEY: np = not present 0 = no staining 0.5 = scanty (+/-)
1 = trace 2 = some
3 = moderate 4 = maximal

Each number separated by a coma (,) represents results from one rat
The 0 hour rats were not injected with PMSG
Glycocalyx = apical surface of luminal epithelium; Secretions = free material in the uterine lumen; Glands = the content and/or lining of the glands
* difference 4 IU PMSG versus 40 IU PMSG versus untreated mature rats ($p<0.05$)
# difference 4 IU versus 40 IU PMSG treated rats ($p<0.001$)
$ difference within 40 IU PMSG Group pre versus post ($p<0.001$)

Table 9d. Results for the "amount" (relative area) of the glycocalyx, uterine secretions and glands, from the middle uterine horn cross-section, as stained by the AB1.0 method

Hours Post PMSG	Glycocalyx 4 IU *	Glycocalyx 40 IU *	Uterine Secretions 4 IU * #	Uterine Secretions 40 IU * #	Glands 4 IU *	Glands 40 IU *
0	1, 1, 1		np, np, np		np, np, np	
24	2, 2, 2	1, .5, 2	2, 2, 1	2, 2, 2	2, 2	1, .5, .5
48	2, 2.5	2, 2	1.5, 1.5	np, np	.5, np	np, np
72	2, 2	2, 2.5, 2	2.5, 2	2, 3, 1.5	1, 2	2, np
96	1, 2, 1.5	2.5, 2.5, 2.5	1, 2, np	2, 2.5, 2.5, 2	1, 1, 1	np, np, np
120	1.5, 2, 1.5	2.5, 2.5, 2.5	1.5, 1.5, np	2, 2.5, 3.5	1, .5, .5	3, 2
144	2.5, 1, 2	2.5, 2.5, 2	np, np, np	2, 2, 2	1, 1.5, 1	np, np, np
168	2.5, 2, 1.5	2.5, 2.5, 2.5	1, 2, 2.5	2.5, 2.5, 2.5	1, .5, 1	2, 2
192	2, 1.5, 2	1.5, 2, 2	1, 1.5, 1.5	3, 1, 3	.5, 1, 1	4, 4
216	1.5, 2, 0	2, 2, 2	0, np, np	4, 3, 2.5	1, 1.5, 0	3, 3, 1

Stage of Cycle	Glycocalyx *	Uterine Secretions * #	Glands
Proestrus	2, 2, 2	1, 2, np	.5, .5, .5
Estrus	2, 2, 2.5, 2.5	1, 1.5, 3	.5, .5, 1.5
Metestrus	2, 2, 2	0, 0, 0	.5, .5, .5
Diestrus	2,1,1	0, 0, 0	.5, 1, 1

KEY: np = not present
0 = no staining 0.5 = scanty (+/-)
1 = trace 2 = some
3 = moderate 4 = maximal
Each number separated by a coma (,) represents results from one rat

The 0 hour rats were not injected with PMSG
Glycocalyx = apical surface of luminal epithelium
Secretions = free material in the uterine lumen
Glands = the content and/or lining of the glands

* difference 4 IU PMSG versus 40 IU PMSG versus untreated mature rats ($p<0.05$)
# difference 4 IU versus 40 IU PMSG treated rats ($p<0.001$)
$ difference within 40 IU PMSG Group pre versus post ($p<0.001$)

Table 9e. Results for the "amount" (relative area) of the glycocalyx, uterine secretions and glands, from the middle uterine horn cross-section, as stained by the PA*S method

Hours Post PMSG	Glycocalyx 4 IU	Glycocalyx 40 IU	Uterine Secretions 4 IU *	Uterine Secretions 40 IU * # $	Glands 4 IU	Glands 40 IU
0	.5, .5		.5, np		np, np	
24	.5, 1, 2	.5, .5, 1.5	1, 1.5	2, 2	1, np	1.5
48	2, 1.5	.5, .5	1, 2	.5, .5	np	.5, 1.5
72	1, 2.5, .5	1, 2, 1.5	3, 2, .5	2, 2, 1	1, 2.5	2.5, 2
96	0, 2	1.5, 2, 2	2.5, 2	1, 2.5, 1	1.5, 1.5	2, 2
120	.5, .5, .5	2, 2, 2	.5, 1	3, 3.5, 2	2, .5	1.5, 1.5
144	.5, .5, 1	2, 2, 2	.5, 1, .5	2, 2.5, 2.5	1, 2.5	1.5, 1.5
168	2, 1.5, .5	1.5, 2, 2	1, 2, 2	2.5, 3, 3	1, 1	1, 1.5, 2
192	1, .5	1.5, 2, 1.5	1, .5	3, 1.5, 2.5	1.5, 2	2, 1.5, 2
216	1, .5, 1	2, 2, 2	np, np, np	3.5, 3.5, 3.5	1.5, 1, 1.5	2, 3, 3

Stage of Cycle	Glycocalyx	Uterine Secretions *	Glands
Proestrus	1.5, 1.5, 1.5	.5, np, 1	np, np, np
Estrus	2, 2, 1.5, 1.5	2, np, 1, 1.5	1.5, 1.5, 2, .5
Metestrus	1.5, 2, 2.5	1, np, np	1.5, 2, 2
Diestrus	1, .5, 2	np, np, np	1, 2, 1.5

KEY:
np = not present
0 = no staining 0.5 = scanty (+/-)
1 = trace 2 = some
3 = moderate 4 = maximal
Each number separated by a coma (,) represents results from one rat
The 0 hour rats were not injected with PMSG
Glycocalyx = apical surface of luminal epithelium
Secretions = free material in the uterine lumen
Glands = the content and/or lining of the glands

* difference 4 IU PMSG versus 40 IU PMSG versus untreated mature rats ($p<0.05$)
# difference 4 IU versus 40 IU PMSG treated rats ($p<0.001$)
$ difference within 40 IU PMSG Group pre versus post ($p<0.001$)

Table 9f. Results for the "amount" (relative area) in the glycocalyx, uterine secretions and glands, from the middle uterine horn cross-section as stained by the PA*/Bh/PAS method

Hours Post PMSG	Glycocalyx 4 IU *	Glycocalyx 40 IU *	Uterine Secretions 4 IU *	Uterine Secretions 40 IU *	Glands 4 IU *	Glands 40 IU *
0	.5, .5		1, .5		np, np	
24	1, 1, 1.5	1, 1, 2	2, 2	3, np, np	np, np	np, np
48	1.5, 1.5	1.5, 1.5	1, 2	1.5, np	1, np	np, np
72	1, 2	2, 2	2.5, 2.5	3, 1.5	2, 2	2, 1.5
96	1, 2, 1	2, 2, 2	2, np, np	1, 2.5, 2	2, 1.5, 1	2, np
120	1, 1.5, 1	2, 2.5, 2.5	2, 1.5, 2	2, 3.5, 2.5	2, 1.5, 2	2, np
144	2, 1.5, 1, 1	2, 1.5, 2, 2.5	2, 1	2, 2, 2.5, 3	1, 1, np	np, np, np
168	2, 1.5, 2	2, 2.5, 3	2, 2	2.5, 3, 2.5, 3	.5, 1	1.5, np
192	1.5, 1.5, 2	1.5, 3, 2	1.5, np, np	2, 1.5, np	1, 1, np	1.5, 2, 3
216	1, 1, 0	3, 3, 2	np, np, np	3, 4, 2.5	1.5, .5, 2	3, 3, 2.5

Stage of Cycle	Glycocalyx	Uterine Secretions	Glands
Proestrus	1, 1, .5	np, 1, np	np, np, np
Estrus	2, 1.5, 1.5, 1.5	2, 2, np	np, 1, 2, 2
Metestrus	2, 2, 2	np, np, 2	1, 1.5, np
Diestrus	1, 1, 1.5	np	np, 1, 1.5

KEY:
np = not present
0 = no staining 0.5 = scanty (+/-)
1 = trace 2 = some
3 = moderate 4 = maximal

Each number separated by a coma (,) represents results from one rat

The 0 hour rats were not injected with PMSG

Glycocalyx = apical surface of luminal epithelium
Secretions = free material in the uterine lumen
Glands = the content and/or lining of the glands
* difference 4 IU PMSG versus 40 IU PMSG versus untreated mature rats ($p<0.05$)

Table 10a. Comparison of the amount and intensity of carbohydrate staining of the glycocalyx in the four segments of the uterus and the statistical differences seen in 4 IU, 40 IU PMSG treated immature and mature rats

CARBOHYDRATE		4 x 40 x mature				4 x 40			
		1	2	3	4	1	2	3	4
Sialic acid	Amount	-	-	-	-	-	-	-	-
	Intensity	X	X	X	-	-	-	-	-
Sulphate esters	Amount	X	X	X	X	-	X	-	-
	Intensity	X	X	X	X	-	-	-	-
Neutral sugars	Amount	X	X	X	X	X	X	-	X
	Intensity	X	X	X	X	X	X	X	X
Ratio Sialic acid: Sulphate esters	Amount	X	X	X	X	X	X	X	X
	Intensity	-	-	-	-	-	-	-	-
Ratio Sulphate esters: Neutral sugars	Amount	X	X	X	X	X	X	X	X
	Intensity	-	X	-	-	-	-	-	-
Ratio Sialic acid: Neutral sugars	Amount	X	X	X	X	X	-	-	X
	Intensity	X	X	X	-	-	-	-	-

Key:
4 = 4 IU PMSG treated immature rats
40 = 40 IU PMSG treated immature rats
mature = untreated mature rats

4 x 40 x mature = all three groups compared
4 x 40 = groups of immature rats compared to each other

1 = oviductal segment of uterine horn
2 = middle segment of uterine horn
3 = cervical segment of uterine horn
4 = cervical vaginal region

amount = relative area of glycocalyx stained
intensity = density of stain

X = significant difference ($p<0.001$)
- = no difference

Table 10b. Comparison of the amount and intensity of carbohydrate staining of the secretions in the four segments of the uterus and the statistical differences seen in 4 IU, 40 IU PMSG treated immature and mature rats

CARBOHYDRATE		4 x 40 x mature				4 x 40			
		1	2	3	4	1	2	3	4
Sialic acid	Amount	X	X	X	X	-	-	-	X
	Intensity	X	X	X	X	X	X	-	-
Sulphate esters	Amount	X	X	X	X	-	X	-	X
	Intensity	X	X	X	X	-	-	-	-
Neutral sugars	Amount	X	X	X	-	-	-	-	-
	Intensity	X	-	X	X	X	-	-	-
Ratio Sialic acid: Sulphate esters	Amount	X	X	X	X	X	X	X	X
	Intensity	-	-	-	-	-	-	-	-
Ratio Sulphate esters: Neutral sugars	Amount	X	X	X	X	-	X	X	X
	Intensity	X	-	-	X	-	-	-	-
Ratio Sialic acid: Neutral sugars	Amount	X	X	X	X	-	X	-	X
	Intensity	X	X	X	-	-	-	-	-

Key:

4 = 4 IU PMSG treated immature rats
40 = 40 IU PMSG treated immature rats
mature = untreated mature rats

4 x 40 x mature = all three groups compared
4 x 40 = groups of immature rats compared to each other

1 = oviductal segment of uterine horn
2 = middle segment of uterine horn
3 = cervical segment of uterine horn
4 = cervical vaginal region

amount = relative area of secretions stained
intensity = density of stain

X = significant difference ($p<0.001$)
- = no difference

Table 10c. Comparison of the amount and intensity of carbohydrate staining of the glands in the four segments of the uterus and the statistical differences seen in 4 IU, 40 IU PMSG treated immature and mature rats

CARBOHYDRATE		4 x 40 x mature				4 x 40			
		1	2	3	4	1	2	3	4
Sialic acid	Amount	X	-	-	-	-	-	-	-
	Intensity	-	-	-	-	-	-	-	-
Sulphate esters	Amount	-	X	X	-	-	-	-	-
	Intensity	X	X	X	-	-	-	-	-
Neutral sugars	Amount	-	X	X	-	-	-	-	-
	Intensity	-	-	-	-	-	-	-	-
Ratio Sialic acid: Sulphate esters	Amount	-	-	-	-	-	-	-	-
	Intensity	X	-	-	-	-	-	-	-
Ratio Sulphate esters: Neutral sugars	Amount	-	-	-	-	-	-	-	-
	Intensity	-	-	X	-	-	-	-	-
Ratio Sialic acid: Neutral sugars	Amount	-	-	-	-	-	-	-	-
	Intensity	-	-	-	-	-	-	-	-

Key:
4 = 4 IU PMSG treated immature rats
40 = 40 IU PMSG treated immature rats
mature = untreated mature rats

4 x 40 x mature = all three groups compared
4 x 40 = groups of immature rats compared to each other

1 = oviductal segment of uterine horn
2 = middle segment of uterine horn
3 = cervical segment of uterine horn
4 = cervical vaginal region

amount = relative area of gland stained
intensity = density of stain

X = significant difference ($p<0.001$)
- = no difference

Table 11. Comparison of the amount and intensity of carbohydrate staining of the glycocalyx and the uterine secretions in the four segments of the uterus within the 40 IU PMSG group ("pre" versus "post")

CARBOHYDRATE		Glycocalyx				Secretions			
		1	2	3	4	1	2	3	4
Sialic acid	Amount	-	-	-	-	-	X	-	-
	Intensity	-	-	-	-	-	X	X	X
Sulphate esters	Amount	-	-	-	-	-	-	-	-
	Intensity	-	-	-	-	-	-	-	-
Neutral sugars	Amount	-	-	X	-	-	-	-	-
	Intensity	X	X	X	-	-	-	-	-
Ratio Sialic acid: Sulphate esters	Amount	X	-	-	-	-	-	-	X
	Intensity	-	-	-	-	-	-	-	-
Ratio Sulphate esters: Neutral sugars	Amount	X	X	X	X	-	-	-	-
	Intensity	-	-	-	-	-	-	-	-
Ratio Sialic acid: Neutral sugars	Amount	-	-	-	-	-	X	-	-
	Intensity	-	-	-	-	-	-	-	-

Key:

1 = oviductal segment of uterine horn
2 = middle segment of uterine horn
3 = cervical segment of uterine horn
4 = cervical vaginal region

X = significant difference ($p<0.001$)
- = no difference

amount = refers to the relative area stained
intensity = density of staining

Table 12a. Summary of the relative amount of uterine secretions & glycocalyx when the 4 IU PMSG treated immature rats and untreated mature rats are compared to the 40 IU PMSG treated immature rats

	4 IU PMSG & Mature	40 IU PMSG
UTERINE SECRETIONS	↔	↑
GLYCOCALYX	↔	↑

Key:

4 IU PMSG = 4 IU PMSG treated immature rats
40 IU PMSG = 40 IU PMSG treated immature rats
Mature = untreated mature rats

↔ = present

↑ = increased

Table 12b. Summary of the carbohydrate composition of the uterine secretions and the differences between the 4 IU PMSG treated immature rats and untreated mature rats as compared to the 40 IU PMSG treated immature rats

SECRETIONS	4 IU PMSG & Mature	40 IU PMSG
O-sulphate esters	↔	↔
neutral sugars	↔	↑
sialic acid	↔	↑

Key:

4 IU PMSG = 4 IU PMSG treated immature rats
40 IU PMSG = 40 IU PMSG treated immature rats
Mature = untreated mature rats

↔ = present

↑ = increased

Table 12c. Summary of the carbohydrate composition of the uterine secretions and the differences between the 4 IU PMSG treated immature rats and untreated mature rats as compared to the 40 IU PMSG treated immature rats

GLYCOCALYX	4 IU PMSG & Mature	40 IU PMSG
O-sulphate esters	↔	↔
neutral sugars	↔	↑
sialic acid	↔	↔

Key:

4 IU PMSG = 4 IU PMSG treated immature rats
40 IU PMSG = 40 IU PMSG treated immature rats
Mature = untreated mature rats

↔ = present

↑ = increased

Table 12d. Summary of the carbohydrate composition of the uterine secretions within the 40 IU PMSG treated immature rats, comparing "pre" (0 - 96 hours) to "post" (120 - 216 hours)

SECRETIONS	40 IU PMSG "PRE"	40 IU PMSG "POST"
O-sulphate esters	↔	↔
neutral sugars	↔	↔
sialic acid	↔	↑

Key:

4 IU PMSG = 4 IU PMSG treated immature rats
40 IU PMSG = 40 IU PMSG treated immature rats
Mature = untreated mature rats

↔ = present

↑ = increased

Table 12e. Summary of the carbohydrate composition of the glycocalyx, within the 40 IU PMSG treated immature rats, comparing "pre" (0 - 96 hours) to "post" (120 - 216 hours)

GLYCOCALYX	4O IU PMSG "PRE"	40 IU PMSG "POST"
O-sulphate esters	↔	↔
neutral sugars	↔	↑
sialic acid	↔	↔

Key:

4 IU PMSG = 4 IU PMSG treated immature rats
40 IU PMSG = 40 IU PMSG treated immature rats
Mature = untreated mature rats

↔ = present

↑ = increased

4.0 DISCUSSION

4.1 Ovulatory response, ovarian and uterine weight

Superovulation using a 40 IU PMSG dose resulted in ovulation which began 24 h earlier and with greater frequency of morphologically normal and abnormal oocytes released then when a dose of 4 IU PMSG was employed. These results are similar to that found by others (Ying et al., 1969; Parker et al., 1976; Leveille et al., 1989; Miller & Armstrong, 1981; Yun et al., 1987, Yun, Yu, Ho Yuen & Moon 1989). It would have been interesting to have flushed the oviducts and uteri from the 4 IU and 40 IU PMSG treated immature rats from 96 h to the end of the experiment, as well as from the mature rats. Knowledge of the ovulatory response and the morphology of the oocytes from the superovulated animals through the entire course of the experiment may have confirmed the interpretation of the steroid hormone response. Also, it may have more clearly shown that the 4 IU PMSG injected rats were cycling in a similar fashion as mature rats, if another set of ovulations were seen.

The significantly higher ovarian weight in the 40 IU PMSG group was attributed to the hyperstimulation of the follicles in the ovary in response to the superovulatory dose of PMSG. The ovarian weights were similar in the 4 IU PMSG group and the mature rats and did not differ during the various stages of the estrous cycle. The uterine weight was higher 24 h after the administration of either a 4 IU or 40 IU dose of PMSG. This may have been one of the first indications of the successful induction of puberty in the immature rat. The substantial increase in uterine weight in the superovulated group may have initially been caused by stromal hypertrophy, but the subsequent increase in uterine weight was likely dependent upon epithelial proliferation and growth.

The changes seen in the ovarian and uterine weights due to the administration of PMSG were similar to those found in other studies (Miller et al., 1981; Parker et al., 1976). The results of the ovulatory response, ovarian and uterine weights indicate that the rats used in this study had the expected responses to the doses of PMSG injected.

4.2 Serum steroid response

This study confirms that a low dose of 4 IU PMSG can induce immature rats to mimic the steroid hormone patterns for estradiol, progesterone and androgens associated with the mature rat estrus cycle. The patterns of progesterone and estradiol concentrations are in fair agreement with those observed by other workers using low doses (4-8 IU) of PMSG (Nuti et al., 1975; Parker et al., 1976) and the adult rat (Wilson et al., 1974).

Previous studies in this laboratory have shown that superovulatory treatment with 20 IU or 40 IU PMSG results in an elevation of progesterone and an initial decrease and subsequent increase in androgens as compared to the 4 IU PMSG treated immature rat (Fang, 1988; Yun et al., 1989). This study, using a superovulatory dose of 40 IU PMSG, gave similar patterns of steroid hormone response. Superovulatory doses of PMSG disrupted the sequential changes of ovarian steroids with progressive increases in the ratios of progesterone compared to either estradiol or androgens in comparison to 4 IU PMSG treated rats or mature rats. A correct ratio of progesterone to estradiol is critical to the establishment of viable pregnancies (Smitz, Devroey & Van Steirteghem, 1990).

4.3 Morphology of the rat uterus

The untreated mature rats and the 4 IU PMSG treated immature rats had cyclic uterine epithelial patterns. Endometrial morphological changes during the rat estrous cycle is controlled by the concomitant fluctuations of estrogen and progesterone secretion (Finn, 1977). During proestrus, the uterus becomes maximally dilated by the accumulation and retention of fluid in the uterine lumen. An increase in luminal epithelial proliferation is associated with the elevation of estrogen levels 24 h prior to maximum dilation (Kennedy & Armstrong, 1975; Finn, 1977). During estrus, the declining estrogen levels and the rising progesterone levels synergistically relax the constricted cervix and allow for the drainage of uterine fluid through the vagina (Kennedy et al., 1975). The uterus then involutes and compresses the endometrium into flaccid folds. When all the serum steroid concentrations return to baseline, there is stromal hypotrophy and epithelial degeneration characterized by nuclear debris and cytoplasmic vacuolation (Finn, 1977).

The 40 IU PMSG treated immature rats in contrast did not exhibit cyclic uterine epithelial patterns which resulted in degenerative activity. Instead, the endometrium became progressively hyperplastic, with focal and diffuse luminal papillary hyperplasia, subepithelial stromal cells disorganization and an increase in the lengths of the inner luminal perimeter. The appearance of the uteri 168 hours post 40 IU PMSG administration, which would correspond to the normal time of implantation, did not resemble the uteri from 4 IU PMSG treated rats or untreated mature rats. Consequently, if a viable blastocyst was present in the uterus of a superovulated rat, normal luminal epithelial cell - blastocyst interactions would probably not occur due to the morphological endometrial abnormalities. Therefore, it is likely that the morphological endometrial changes in the superovulated rat contribute to their decreased pregnancy rates.

4.3.1 Effect of serum steroids on the endometrium

The hormonal pattern of estradiol and progesterone in the 4 IU PMSG treated immature rats and the mature rats were similar. Therefore, the correspondence of the uterine morphology was not surprising. However, the serum concentrations of estradiol, progesterone and androgens were increased in immature rats following treatment with a superovulatory dose of 40 IU PMSG. The disruption of the hormonal pattern of estradiol, progesterone and androgens in the 40 IU PMSG treated immature rats probably lead to the abnormal uterine morphology and carbohydrate histochemistry seen in this study.

4.3.1.1 Effect of estradiol

Endogenous and exogenous estradiol initiate hypertrophy of the endometrium through the development of hyperemia (of the vascular system) and edema in the subepithelial stroma (Galand, Leroy, Chretien, 1971). Time course studies of ovariectomized rats given a single dose of estradiol reveal that the uterine weight and volume, subepithelial stromal edema and epithelial proliferation rates rapidly develop to a dose dependent maximum between 6-10 hours, but then regresses within 20 hours (Carroll, 1945). However, if a second injection of the same dose of estradiol was given one day later, the magnitude of all these parameters rapidly increased to a new and higher maxima within 16-24 h (Martin, Finn & Trinder, 1973). These results from a single and double injection of estradiol appear to parallel the observations seen in the 40 IU PMSG rats in this study. The presence of the stromal hypertrophy observed 72 hours after 40 IU PMSG treatment may not be related only to the elevated estradiol concentrations 48 h post PMSG injection but also due to the increased estradiol concentrations present throughout the course of the experiment.

An interesting morphological feature in this study was the focal and diffuse luminal papillary hyperplasia seen after the administration of 40 IU PMSG. Hypertrophy of the

subepithelial stroma occurred before the development of hyperplasia of the luminal epithelium. It is possible that the prolonged and elevated levels of estradiol from 24 to 48 h initiated this sequential pattern. Similar patterns have been observed in ovariectomized rodents repeatedly treated with estrogens (Carrol, 1945; Finn et al., 1977).

The epithelial hyperplasia was initially focal and appeared in the antimesometrial region of the uterus. This focal appearance of the luminal epithelium may have been the result of the dissimilar hormonal sensitivities between the anti-mesometrial and mesometrial regions of the uterus. Autoradiographic studies of ovariectomized mice treated with a single dose of estradiol have shown that all regions of the uterus are equally radiolabeled for up to 16 h. However, after this time, the rate of radiolabelling in the mesometrial area of the uterus decline, while those of the antimesometrial region remained elevated for up to 36 h (Martin et al., 1973). The prolonged and high concentrations of serum estradiol associated with the superovulatory treatment may have exaggerated the existing elevated proliferation rates in the antimesometrial region and contributed to the formation of the focal hyperplasia seen in the 40 IU PMSG treated uteri after 96 hours. It is also possible that the earlier elevations of estradiol (at 24-48 hours) primed the epithelial cells to respond more intensely to a second stimulus. The changes induced by this second stimulus were then superimposed over those elicited by the first exposure to estradiol. Estrogen may effect cell proliferation indirectly by paracrine interactions between the luminal epithelium and the subepithelial stromal cells. The subepithelial stromal cells produce Interleukin-6 (IL-6) which inhibits epithelial cell replication. Estradiol action on the subepithelial stroma reduces the amount of stromal cell IL-6 produced and may indirectly enhance epithelial cell growth by reducing the levels of a stromal factor that normally restrains epithelial cell proliferation (Strauss, 1991).

4.3.1.2 Effect of progesterone

Progesterone is essential for the development of endometrial sensitivity, in the rat, but estrogen secretion is also required immediately before the time of implantation. The ability of progesterone to regulate estrogen action in the mammalian uterus is crucial in establishing and maintaining reproductive competency (Leavitt, 1989). Progesterone is essential for the maintenance of pregnancy in all mammalian species. Failure of implantation is an important cause of infertility and limits the success of treatments based on fertilization in vitro and embryo transfer (Bullock et al., 1986).

Progesterone influences morphological changes in the subepithelial stromal cells. The most prominent stromal changes occur in the nuclei (especially the nucleoli), with increased activity also in the cytoplasm; including an increase in rough endoplasmic reticulum and free ribosomes. However, full differentiation of the stromal cells, involving a transformation into decidual cells under the influence of hormones alone does not occur in the rat (Finn, 1977).

The appearance of intra-luminal secretory material with diffuse hyperplasia in 40 IU PMSG treated rats may have been coincidental and not related to the presence of hyperplasia. However, the presence of copious amounts of secretions follows the prolonged exposure to progesterone. Progesterone influences the production of some major uterine proteins which include: Uteroglobin (endometrial protein which is a constituent of uterine fluid before implantation in the rabbit) and progestin-dependent endometrial protein (PEP), a glycoprotein. The uterus of the pig secretes large amounts of protein in response to progesterone (Roberts, Murray, Burke, 1986). The responses of the uterus to progesterone require prolonged hormone treatment and are not immediate.

4.3.1.3 Effect of androgen

The uteri in the "post" stage from 40 IU PMSG treated rats was hypertrophic, the endometrium was hyperplastic and the subepithelial stroma was disorganized. Exogenous aromatizable and non-aromatizable androgens may induce a sustained hypertrophy of the uterus for up to 72 h after treatment (Armstrong, Moon & Leung, 1976; Rennels et al., 1977). Androgens may act directly upon the uterus through their own receptors, or if there are elevated peripheral androgen concentrations, act through the estradiol receptors (Lobel, Liliane & Shelesnyak, 1977). Thus androgens may have induced the uterine epithelial hypertrophy through protein and carbohydrate synthesis associated with growth (Armstrong et al., 1976).

Despite the sharp fall in serum estradiol concentrations 60 h post 40 IU PMSG injection, these estradiol concentrations were still higher than those found in the 4 IU PMSG rats. Estradiol in combination with elevated androgens may have provided sufficient support to account for the absence of endometrial degeneration and repair. It is possible that estradiol and androgens may have synergistically induced and maintained the hypertrophy of the stroma for an extended period of time.

4.3.2 Mitotic figures

Mitotic rates did not correlate well with the changes seen in the length of the luminal perimeter. Also, the development of focal hyperplasia beginning at 72 h in the 40 IU PMSG treated rats was not accompanied by an increase in the number of mitotic figures. However, it is possible that the mitotic divisions were rapid, highly synchronized and occurred between the time of sacrifices.

It is well established that estrogens increase the proliferation rate through the

shortening of the G1 and S phases of the cell cycle (Leroy, Galand & Chretien, 1969; Martin et al., 1973). Stimulation of the cell with estrogen promotes a synchronized entry into the S and the mitosis phases. Exogenous estrogen stimulation in ovariectomized rodents have induced cell cycle lengths of less than 15 h; with a mitotic rate of 1 h in the uterine luminal epithelial cells (Martin et al., 1973). Bullough (1950) used Bouin's fixative and observed a progressive reduction in the number of cells during each phase of mitosis for up to 3 h, indicating that the cessation of mitotic activity was dependent upon fixation penetration and not on the time of sacrifice. Thus it would have been possible for proliferative changes initiated soon after a sacrifice to finish prior to the completion of fixation or the next sacrifice. Therefore, even though the uterine tissue used in this study was immediately placed in Bouin's fixative, cell division may not have been instantly arrested.

4.3.3 Uterine luminal perimeters

The inner luminal epithelium is much more sensitive to hormonal influences then either the glandular epithelium or the subepithelial stromal tissue (Martin et al., 1973; Marcus, 1974). The effects of the steroid hormones on the endometrium are dependent upon the sensitivity and proportion of glandular and luminal epithelial tissue (Arias-Stella, 1954; Meissner, Sommers & Sherman, 1957). In the rat there is proportionally less glandular epithelium than luminal epithelium. This feature may help account for the presence of the increased proliferation in the luminal epithelium but the absence of abnormal proliferation in the glandular tissue.

In addition, the differential hormonal sensitivities of the luminal epithelium and subepithelial stroma may also help explain the papillary appearance of the hyperplastic endometrium. Since the luminal epithelium grows faster than the subepithelial stroma, the endometrium is forced to fold to allow for an increase in surface area within a restricted

volume. If there had been synchronized growth between the subepithelial stroma and the luminal epithelium, the endometrium would be less convoluted, and be similar in appearance to that observed during the onset of proestrus in the mature rat.

In this study, there was a time and dose dependent change in the length of the uterine luminal perimeters. A significant increase in the uterine luminal perimeters in the superovulatory group may be equated with an increase in epithelial proliferation. The results for progesterone, endometrial morphology, number of mitotic figures and lumen perimeters were similar to that found in a previous study in this laboratory (Fang, 1988).

4.4 Carbohydrate histochemistry

The term "histochemistry" implies the ability to carry out chemical reactions on tissues and the subsequent ability to identify under the microscope the localized reaction products. If the chemical reaction is specific, it should be possible to infer the chemical composition of these regions of the tissue (Luft, 1976). In this study, histochemical methods demonstrated the presence of a mixture of carbohydrates in all sections of rat uterus. These carbohydrates included: neutral sugars, O-sulphate esters and sialic acids without side chain O-acyl substituent. The neutral sugars were alpha amylase resistant. Consequently glycogen was not present. This finding is similar to that found previously in this laboratory (Fang, 1988) and by others (Staneva, 1990). The neutral sugars have periodate oxidizable vicinal diols, but were not further identified.

There was a cyclical pattern in the amount of glycocalyx and the secretions in the 4 IU PMSG treated immature rat and the mature rat uterus. The glycocalyx and secretions in the mature rat were most abundant at proestrus and estrus and were sparse at diestrus. These findings agree with the prevailing opinion regarding the stimulatory action of the

estrogens on biosynthesis and secretion in the endometrium (Staneva, 1990). There was a similar cyclical pattern in the 4 IU PMSG treated immature rat over the course of the experiment. This suggests that the 4 IU PMSG treated immature rat is similar to the untreated mature rat.

Increased quantities of sulphate esters, neutral sugars and especially sialic acid were observed in the secretions in the uterine lumen of the superovulated rats, in particular during the "post" stage (secretory phase). The glycocalyx on the luminal epithelial cells in superovulated rats was differed from the uterine secretions. The carbohydrate composition of the superovulated rat glycocalyx showed an increase in the amount of neutral sugars, especially during the "post" stage. In contrast, the cervical mucin during the secretory phase in the human female has an increased ratio of sulphated acidic sugars to sialic acids (Gilks et al., 1988). However, in the 4 IU PMSG treated immature rat and the mature rat, the ratio of sulphated sugars to sialic acids does not differ.

Large quantities of acid mucopolysaccharide may be produced and secreted by the endometrium and certain segments (isthmus, ampulla) of the oviduct in the rat, mouse and rabbit under the influence of estrogen (Finn, 1977). Although statistical comparisons were not done to compare the various sections of the uterus (oviductal, middle, cervical horn as well as the cervix-vaginal region) there did not appear to be any visual staining differences in the distribution of sialic acid, sulphate esters or neutral sugars between the different sections of the rat uterus.

The changes in the patterns of glycoprotein expression by uterine epithelial cells are of particular interest since it is the cell surface of these cells that mediates the initial phase of embryo-uterine attachment. Histochemical studies have shown that pattern of glycoconjugate expression at the luminal (apical) surfaces of these cells change during the

conversion from a non-receptive to a receptive uterine state (Carson et al., 1989). One consistent observation is that the negative charge characteristic of these surfaces decreases when in the receptive state (Aplin, 1989). Another consistent observation is that the conversion to a receptive uterine state is accompanied by increases in the expression of N-acetylglucosaminyl and galactosyl residues (Aplin, 1989). This study has found that there may actually be an increase in the amount of negatively charged substances (sialic acid) in the uterine secretions in the superovulated group. Since a decrease in charge appears to be necessary for a receptive uterus, this may be a important reason that superovulated rats are subfertile.

Sulphated glycoproteins were minimally present in the stromal ground substance and on the collagen, but were more prevalent and localized in the glycocalyx and secretions in the estrous rat endometrium. These observations correlate well with earlier histochemical results on the stimulatory effect of estradiol on the synthesis of acid sulphated mucopolysaccharide in the endometrium of ovariectomized rats and rabbits. Pharmacological doses of estrogen induce endometrial stromal production of acid mucins that are subsequently transudated through the epithelium into the lumen (Zachariae, 1958). Uterine epithelial mucin production can be initiated by progesterone if adequate priming with estrogen has occurred. The presence of the intra-luminal secretory material (present between 96-192 h) may represent a latent effect of the earlier elevated estradiol levels and an immediate effect of rising progesterone levels, resulting in production and secretion from the subepithelial stroma and the luminal epithelium. There is also the possibility that some or most of the secretory material originated from the oviduct. The intensification of alcian blue staining by 144 h in the superovulatory treated rats may have resulted from the leakage of excess sulphated mucins into the uterine lumen.

It is not possible to provide an explanation for the induction of mucification (increase in the uterine secretions) that will unify all potential causative factors. Mucification has been previously described in the 10 and 15 d old rat following superovulatory treatment with PMSG (Rennels et al., 1977; Parlanti & Monis, 1980). In ovariectomized rats, Rennels achieved comparable results with exogenous androgens and proposed that androgen stimulation may have been an underlying action of PMSG. Studies with adrenalectomized and ovariectomized rats demonstrated that non and aromatizable androgens are capable of inducing mucification (Kennedy & Armstrong 1976).

High levels of prolactin may have also been a causative factor of mucification. A study using a 50 IU dose of PMSG revealed increases in the serum prolactin concentrations, likely through the action of estrogens or aromatizable androgens on the pituitary (Rennels et al., 1977). Other studies have revealed that prolactin stimulated vaginal mucification via an extra-ovarian action. However, mucification depended upon the synergistic actions of both estrogen and progesterone (Kennedy & Armstrong, 1972, 1973). Progesterone induces mucinogenic differentiation (Bertalanfly & Lau, 1963) and the elevated progesterone concentrations from 168-240 h post 40 IU PMSG treatment may help explain the excessive mucification present. The increased serum androgen concentrations seen may also have an important role.

The results of this study suggest that superovulatory doses of PMSG induce pathological structural and metabolic alterations in the uterus. Although apparently reversible, many of these effects persist past the time of normal implantation. Therefore, it is possible that the effects induced by superovulation interfere with the pre-implantational preparation of the uterus. Alternatively, changes in the glycocalyx may actively interfere with the implantation process.

5.0 CONCLUSIONS

The administration of a 4 IU PMSG dose in an otherwise immature rat produced ovulatory patterns of serum estradiol, progesterone, androgen concentrations (and ratios), and uterine morphological changes similar to that found in a normal estrous cycle in the untreated mature rat.

In contrast, the administration of a 40 IU PMSG (superovulatory) dose produced an increased number of (both morphologically normal and abnormal) oocytes, along with high levels of serum estradiol progesterone and androgens. Superovulation was associated with endometrial hyperplasia, disorganization of the subepithelial stroma and an increase in the uterine lumen perimeter.

The selective carbohydrate histochemical methods used in this study indicated that the 4 IU PMSG dose produced patterns and amounts of sialic acid, sulphate esters and neutral sugars that were similar to that found in the mature rat. The PAS positive staining material was alpha amylase resistant; indicating that glycogen was not present.

The administration of a 40 IU dose of PMSG was associated with an increased amount of free secretions and glycocalyx, which was different from both the 4 IU PMSG treated immature rats and the mature rats. The free secretions in the 40 IU PMSG rats stained more intensely for sialic acid and neutral sugar. The glycocalyx in the superovulatory group stained differently then the secretions. In the glycocalyx of the 40 IU PMSG immature rats, the neutral sugars stained more intensely then in the 4 IU PMSG treated and mature rats. There was an increase in the amount of the uterine secretions and in the glycocalyx during the "post" phase, which coincided with higher progesterone concentrations. There was increased staining for sialic acid in the secretions and for neutral sugars in the glycocalyx

during the "post" phase.

The disruption of the normal balance of steroid hormones in the superovulatory 40 IU PMSG group likely caused the abnormal morphological changes seen in the endometrium. These changes may be disruptive to successful apposition of the blastocyst to the uterine wall. The increase in secretions in the lumen may act as both a physical barrier to adhesion and/or prevent effective blastocyst-uterine epithelial cell communication. The increase in sialic acid seen in the secretions may disrupt the changes in charge associated with implantation. These abnormal changes may combine to prevent successful implantation and therefore contribute to the decrease in the fertility rate seen in the superovulated immature rat.

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7.0 APPENDIX ONE

7.1 Description of slides

Slides 1 - 24 are light micrographs of Bouin's fixed paraplast embedded rat uteri. The surface epithelium of the uterine lumen is to the top and the subepithelial stroma is to the bottom. The 4 micron thick cross-sections from the middle uterine horn segment were photographed at a magnification of 500X.

Slide 1. Proestrus, mature rat, stained with PA*/T/Bh/PAS/KOH. The luminal epithelial glycocalyx and the secretions stained red-purple, indicating the presence of both sialic acid and neutral sugars. However, the gland is not stained. Note the intense red staining of the subepithelial stroma due to the presence of neutral sugars.

Slide 2. Estrus, mature rat, stained with PA*/T/Bh/PAS/KOH. The luminal epithelial glycocalyx stained red-purple, indicating the presence of both sialic acid and neutral sugars. Note that there is also red-purple stained material present in the glandular lumen.

Slide 3. Metestrus, mature rat, stained with PA*/T/Bh/PAS/KOH. The luminal epithelial glycocalyx and the secretions stained red-purple, indicating the presence of both sialic acid and neutral sugars. The glycocalyx of the glandular epithelial cells stained purple.

Slide 4. Diestrus, mature rat, stained with PA*/T/Bh/PAS/KOH. The luminal epithelial glycocalyx stained purple, indicating the presence of both sialic acid and neutral sugars. Note that the gland is again unstained as compared to estrus or metestrus (slide 2 and 3).

Slide 5. 30 day old immature rat, 48 h post 4 IU PMSG, stained with PA*/T/Bh/PAS/KOH. The luminal epithelial glycocalyx stained purple-red, indicating the presence of both sialic acid and neutral sugars. There is purple stained material present in the cytoplasm of the glandular epithelial cells. This is similar to metestrus (slide 3).

Slide 6. 30 day old immature rat, 48 h post 40 IU PMSG, stained with PA*/T/Bh/PAS/KOH. The luminal epithelial glycocalyx stained purple, indicating the presence of both sialic acid and neutral sugars. The uterine secretions stained red-purple. The glandular lumen contains red coloured material.

Slide 7. 35 day old immature rat, 168 h post 4 IU PMSG, stained with PA*/T/Bh/PAS/KOH. The luminal epithelial glycocalyx stained purple, indicating the presence of both sialic acid and neutral sugars. There is an increased amount when compared to 4 IU PMSG at 48 h (slide 5). The apical surfaces of the glandular epithelium also stained purple. This is similar to estrus in the mature rat (slide 2).

Slide 8. 35 day old immature rat, 168 h post 40 IU PMSG, stained with PA*/T/Bh/PAS/KOH. The luminal epithelial glycocalyx stained blue-purple, indicating the presence of sialic acid and neutral sugars. There increase in the amount of free secretions when compared to any other time or stage ($p<0.001$) (slides 1 - 7).

Slide 9. Proestrus, mature rat, stained with PA*/AB1.0/S. The luminal epithelial glycocalyx and secretions stained blue, indicating the presence of both sialic acid and sulphate esters. The glandular epithelial glycocalyx is not stained. Note that the sub-epithelial stroma is unstained, indicating the selectivity of the method for sialic acid.

Slide 10. Estrus, mature rat, stained with PA*/AB1.0/S. The luminal epithelial glycocalyx stained blue, indicating the presence of both sialic acid and sulphate esters. However, now there is red stained (sialic acid) material present in the glandular lumen.

Slide 11. Metestrus, mature rat, stained with PA*/AB1.0/S. The luminal epithelial glycocalyx and secretions stained blue-purple, indicating the presence of both sialic acid and sulphate esters. The glandular epithelial glycocalyx is stained blue with red coloured specks in the cytoplasm.

Slide 12. Diestrus, mature rat, stained with PA*/AB1.0/S. The glycocalyx of both the uterine lumen and glands stained blue-purple, indicating the presence of both sialic acid and sulphate esters.

Slide 13. 30 day old immature rat, 48 h post 4 IU PMSG, stained with PA*/AB1.0/S. The luminal epithelial glycocalyx stained blue, indicating the presence of both sialic acid and sulphate esters. There is purple coloured material present in the glandular lumen. This is similar to estrus (slide 10).

Slide 14. 30 day old immature rat, 48 h post 40 IU PMSG, stained with PA*/AB1.0/S. Sialic acid and sulphate esters staining is similar to that found in the 30 day old immature rat, 48 h post 4 IU PMSG (slide 13).

Slide 15. 35 day old immature rat, 168 h post 4 IU PMSG, stained with PA*/AB1.0/S. The luminal epithelial glycocalyx stained blue, indicating the presence of both sialic acid and sulphate esters. There is an increased amount of glycocalyx compared to 4 IU PMSG at 48 h (slide 13). The glandular glycocalyx also stained blue.

Slide 16. 35 day old immature rat, 168 h post 40 IU PMSG, stained with PA*/AB1.0/S. The luminal epithelial glycocalyx stained blue, indicating the presence of both sialic acid and sulphate esters. There is an increase in the amount of free secretions in the uterine lumen when compared to any other time or stage ($p<0.001$) (slides 9 - 15). These secretions stained purple-blue. The lumen of the gland contains a large amount of a purple-red stained substance (predominately sialic acid).

Slide 17. Proestrus, mature rat, stained with PA*/Bh/AB1.0/PAS. The luminal epithelial glycocalyx and the secretions stained blue-purple, indicating the presence of both sulphate esters and neutral sugars. However, the gland is not stained. Note the intense red colour of the subepithelial stroma due to the presence of neutral sugars.

Slide 18. Estrus, mature rat, stained with PA*/Bh/AB1.0/PAS. The luminal epithelial glycocalyx stained blue-purple, indicating the presence of both sulphate esters and neutral sugars. There is now red-purple stained material present in the glandular lumen.

Slide 19. Metestrus, mature rat, stained with PA*/AB1.0/PAS. The luminal epithelial glycocalyx and the uterine secretions stained blue-purple, indicating the presence of both sulphate esters and neutral sugars. The glandular glycocalyx stained purple-blue with also some red-purple stained patches present.

Slide 20. Diestrus, mature rat, stained with PA*/Bh/AB1.0/PAS. The luminal epithelial glycocalyx and the glands stained blue-purple, indicating the presence of both sulphate esters and neutral sugars. Note that the gland is unstained.

Slide 21. 30 day old immature rat, 48 h post 4 IU PMSG, stained with PA*/Bh/AB1.0/PAS. The luminal epithelial glycocalyx stained blue-purple, indicating the presence of both sulphate esters and neutral sugars. There is red stained material (neutral sugars) present as speckles in the cytoplasm of the glandular epithelial cells as well as red-purple secretions in the lumen of the gland. This is similar to estrus (slide 18) or metestrus (slide 20).

Slide 22. 30 day old immature rat, 48 h post 40 IU PMSG, stained with PA*/Bh/AB1.0/PAS. The luminal epithelial glycocalyx stained a blue-purple colour, indicating the presence of both sulphate esters and neutral sugars. The uterine lumen secretions stained a purple-red colour, while the glandular lumen contained red coloured (neutral sugars) material. This is similar to estrus (slide 18), metestrus (slide 19) and 30 day old immature rat, 48 h post 4 IU PMSG (slide 21).

Slide 23. 35 day old immature rat, 168 h post 4 IU PMSG, stained with PA*/Bh/AB1.0/PAS. The luminal epithelial glycocalyx stained blue-purple, indicating the presence of both sulphate esters and neutral sugars. There is an increased amount when compared to 4 IU PMSG at 48 h (slide 21). The glandular epithelial glycocalyx stained blue-purple. This is similar to estrus (slide 18).

Slide 24. 35 day old immature rat, 168 h post 40 IU PMSG, stained with PA*/Bh/AB1.0/PAS. The luminal epithelial glycocalyx stained blue-purple, indicating the presence of both sulphate esters and neutral sugars. There was an increase in the amount of luminal free secretions when compared to any other time or stage ($p<0.001$) (slides 17 - 23).

Figure 30. Quotation

Johann Wolfgang von Goethe

Opening monologue: Faust Part one

Da steh' ich nun, ich armer Tor

Und bin so klug als wie zuvor!

Bilde mir nicht ein, was Rechts zu wissen,

Bilde mir nicht ein, ich könnte was lehren