THE EFFECTS OF SUPEROVULATION ON OVARIAN FUNCTION AND EMBRYO DEVELOPMENT IN RATS

By

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We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
August 10, 1989
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Date August 17, 1989
ABSTRACT

This study examined primary defects and related mechanisms leading to impaired fertility following superovulation with exogenous gonadotropin. Experiments were designed to address the ovulatory response, oocyte quality (gross morphology and nuclear maturation) and embryo development with reference to ovarian steroid and circulating LH levels in immature rats treated with 4, 20 or 40 IU pregnant mare's serum gonadotropin (PMSG).

Superovulatory doses (20 and 40 IU) of PMSG induced the first ovulation as early as 24 hr and prolonged multiple ovulations with two increases: one before 36 hr and the other after 48 hr, while a low dose (4 IU) of PMSG induced a burst of ovulation between 60 and 72 hr. The biphasic superovulatory response was paralleled with two distinct luteinizing hormone (LH) peaks in circulating blood. Early ovulation could be, in part, due to the exogenous LH activity derived from high doses of PMSG. The initial prolonged elevation of serum LH before 54 hr resulted from actual cross-reaction of the injected PMSG with LH antibody in the assay and was dose-dependent, while a precipitous second elevation between 54 and 60 hr resulted primarily from an endogenous LH surge. Additionally, the observation of a marked ovulation inhibition by using a progesterone antagonist (RU 486) and an estrogen antagonist (tamoxifen) indicated the active participation of progesterone as well as estrogen in the PMSG-induced ovulation.

A dose-dependent increase in the percentage of degenerate oocytes following superovulation was noticed from 36 hr onwards and positively correlated with the timing of increased levels of ovarian and serum
androgens. Furthermore, superovulated oocytes with even normal appearance displayed substantially different stages of nuclear maturation varying from prophase I to metaphase II, while a majority of control oocytes (4 IU PMSG) was synchronized at metaphase II. The incidence of meiotically aberrant oocytes in superovulated rats was closely associated with abnormal follicular steroidogenesis, i.e. a marked alteration of follicular contents of progesterone and particularly androgens and/or a consistent disruption of sequential changes in overall ratios of androgens/17β-estradiol, progesterone/17β-estradiol and progesterone/androgens.

Administration of an androgen antagonist (flutamide) in superovulated rats significantly reduced the proportion of degenerate oocytes and cellular degeneration of preimplantation embryos, and improved the developmental potential (embryo cleavage), at least in part, via decreased production of ovarian androgens. These results reflect a significant role of augmented ovarian androgen secretion in the perturbation of oocyte quality and subsequent embryo development following superovulation. However, the pharmacological effects of flutamide were restricted to early stage (Day 2) of pregnancy. Therefore, the actual improvement of the quality or development of early embryos by flutamide was ascribed to a substantial reduction of abnormal oocytes before fertilization.

The results of an experiment using the RU486 and tamoxifen indicated that estrogen, but not progesterone, influenced oocyte quality in superovulated rats. RU486 treatment did not affect the oocyte morphology, but tamoxifen treatment was associated with a marked increase in the percentage of degenerate oocytes.
The results of this research provide direct evidence of atypical ovulations of superovulated oocytes with premature or asynchronous nuclear maturation as a primary defect leading to impaired fertility including abnormal embryo development, and demonstrate a close relationship between meiotically aberrant oocytes and abnormal follicular steroidogenesis in superovulated rats. Increased levels of ovarian and circulating androgens preceding fertilization were particularly implicated in the perturbation of oocyte quality and embryo development following superovulation. Finally, the process of multiple ovulations induced by superovulatory doses of PMSG appears to involve the exogenous LH activity of PMSG as well as active participation of progesterone and estrogen.
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<td>--------------</td>
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<tr>
<td>B/Bo</td>
<td>Antigen binding ratio per maximum binding</td>
<td></td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
<td></td>
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<tr>
<td>14C</td>
<td>A radioactive isotope of carbon</td>
<td></td>
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<tr>
<td>Ci</td>
<td>Curie (= 3.7x10^{10} disintegrations per second)</td>
<td></td>
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<tr>
<td>CIN</td>
<td>Color index number</td>
<td></td>
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<tr>
<td>CL</td>
<td>Corpora lutea</td>
<td></td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
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<tr>
<td>cpm</td>
<td>Radioactive counts per minute</td>
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</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
<td></td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate-buffered saline</td>
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<tr>
<td>dpm</td>
<td>Radioactive disintegrations per minute</td>
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<tr>
<td>e.g.</td>
<td>For example (exempli gratia)</td>
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<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
<td></td>
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<tr>
<td>g</td>
<td>Gram</td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
<td></td>
</tr>
<tr>
<td>3H</td>
<td>Tritium, a radioactive isotope of hydrogen</td>
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<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin</td>
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<tr>
<td>hMG</td>
<td>Human menopausal gonadotropin</td>
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<tr>
<td>hr(s)</td>
<td>Hour(s)</td>
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<tr>
<td>125I</td>
<td>A radioactive isotope of iodine</td>
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<tr>
<td>i.d.</td>
<td>Inner diameter</td>
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</tr>
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<td>i.e.</td>
<td>That is (id est)</td>
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<tr>
<td>IU</td>
<td>International unit</td>
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<tr>
<td>IVF</td>
<td>In vitro fertilization</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>kg</td>
<td>Kilogram</td>
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<tr>
<td>L:D</td>
<td>Light:Dark, e.g. 12L:12D = 12 hours light:12 hours dark</td>
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<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
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<tr>
<td>LHRH</td>
<td>LH releasing hormone</td>
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<tr>
<td>mCi</td>
<td>Millicurie</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>min</td>
<td>Minute</td>
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<td>ml</td>
<td>Millilitre</td>
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<td>mm</td>
<td>Millimetre</td>
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<tr>
<td>mmol</td>
<td>Millimole</td>
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<tr>
<td>n</td>
<td>Number</td>
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</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
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</tr>
<tr>
<td>NSB</td>
<td>Non-specific binding</td>
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<tr>
<td>OAAD</td>
<td>Ovarian ascorbic acid depletion</td>
<td></td>
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<tr>
<td>pg</td>
<td>Picogram</td>
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<tr>
<td>pH</td>
<td>-Log $H^+$ concentration in a solution</td>
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</tr>
<tr>
<td>Phe</td>
<td>Phenylalanine</td>
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<tr>
<td>PLSD</td>
<td>Protected least significant difference</td>
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<tr>
<td>p.m.</td>
<td>Afternoon (post meridiem)</td>
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<td>PMSG</td>
<td>Pregnant mare's serum gonadotropin</td>
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<tr>
<td>Pro</td>
<td>Proline</td>
<td></td>
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<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
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<td>rpm</td>
<td>Revolutions per minute</td>
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<tr>
<td>SE</td>
<td>Standard error</td>
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<td>sec</td>
<td>Second(s)</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
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<tr>
<td>SEM</td>
<td>Standard error of means</td>
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<tr>
<td>Ser</td>
<td>Serine</td>
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</tr>
<tr>
<td>Sp. act.</td>
<td>Specific activity</td>
<td></td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
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</tr>
<tr>
<td>TSH</td>
<td>Thyroid stimulating hormone</td>
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</tr>
<tr>
<td>v:v:v</td>
<td>Volume ratio</td>
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<td>vs</td>
<td>Versus</td>
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<tr>
<td>µl</td>
<td>Microlitre</td>
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<tr>
<td>µm</td>
<td>Micrometre</td>
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</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
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</tr>
<tr>
<td>Δ</td>
<td>Change in two values</td>
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INTRODUCTION

Induction of superovulation with exogenous gonadotropins is a well-established technique in the livestock industry for enhanced commercial production of genetically superior individuals and in human IVF and embryo replacement programmes for increased yield of oocytes. This technique has also been utilized to facilitate basic studies in reproductive biology including ovulation, fertilization, embryo development and implantation. Despite its widespread application, the use of superovulatory doses of gonadotropin has been reported to result in reduced fertility in large domestic animals (Betteridge and Moore, 1977; Evans and Robinson, 1980; Armstrong and Evans, 1983) and in small laboratory animals (Adams, 1960; Beaumont and Smith, 1975; Miller and Armstrong, 1981a). The impairment is characterized by the highly variable yield of viable embryos, and the frequent recovery of retarded or abnormal embryos (Betteridge, 1977).

Results of numerous studies using immature rats indicate that low doses (4-8 IU) of PMSG induce a synchronized ovulation within 72 hr through eliciting an endogenous LH surge between 54 and 57 hr after injection of the gonadotropin (Sorrentino et al., 1972; Kostyk et al., 1978) and produce normal pregnancy without an increased embryonic or fetal wastage (Nuti et al., 1975). The patterns of circulating steroid hormones (17β-estradiol and progesterone) and LH during the 24 hr preceding 72 hr ovulation (Meyer et al., 1971; Wilson et al., 1974) and the temporal relationship between LH levels and oocyte maturation (Hillensjo et al., 1974) in this regimen are comparable to those observed on the days of proestrus and estrus in cycling adult rats (Barraclough et al., 1971; Linkie and Niswender, 1972). Thus,
PMSG-treated immature rat is a model well suited for studying the mechanisms and effects of superovulation. In immature rats, a single injection of superovulatory doses (16-40 IU) of PMSG leads to a great reduction in fertilization rates (Walton et al., 1983), a substantial loss and degeneration of preimplantation embryos (Miller and Armstrong, 1981b), and a partial or complete failure of implantation (Miller and Armstrong, 1982). This impairment has previously been attributed to the variability in timing of ovulation (Evans and Armstrong, 1983; Walton et al., 1983), excessive stimulation of the genital tract (Miller and Armstrong, 1981b) and resultant asynchrony between embryonic and uterine development by disturbances in the oviductal and uterine environments (Walton and Armstrong, 1982). However, the major factor(s) and critical time involved in the low fertility following superovulation are not fully understood.

Primary defects could occur in the process of oocyte maturation and ovulation prior to fertilization by hyperstimulation of ovarian tissues or acceleration of follicular development because of the prolonged biochemical action of PMSG reported by Schams et al. (1978). Superovulatory dose of PMSG in immature rats has been found to induce an unusually precocious and prolonged ovulatory response (Walton et al., 1983). It appears that a portion of superovulated oocytes is derived from abnormal follicles, which would not ovulate at a regular cyclic estrus (Braw and Tsafriri, 1980; Fleming, 1982). Administration of superovulatory doses of PMSG to sheep alters the pattern of steroid hormones secreted by the follicles cultured in vitro and results in premature activation of oocytes during maturation (Moor et al., 1985). In addition, alterations in the normal pattern of steroid
secretion during follicular maturation induce intracellular changes in sheep oocytes expressed as gross abnormalities at fertilization (Moor et al., 1980b). It has been suggested that follicular steroid microenvironment is a key factor determining follicular growth and oocyte maturation (McNatty and Baird, 1978).

This study was done in an attempt to examine the primary defects and possible mechanisms related to infertility in immature rats following superovulation with PMSG. Experiments were carried out to investigate:

1. Time course of ovulatory response and oocyte morphology with reference to steroid hormone levels in ovarian tissues and peripheral blood during different time periods of treatments with 4, 20, or 40 IU PMSG;

2. Follicular steroid contents and oocyte maturation following PMSG treatments;

3. Circulating LH response to PMSG treatments;

4. Effects of steroid antagonists (flutamide, RU486 and tamoxifen) on ovulatory response and oocyte morphology and/or developmental potential of preimplantation embryos after treatments with PMSG.
REVIEW OF LITERATURE

I. Induction of Superovulation.

More than 60 years ago, the discovery of pituitary and placental gonadotropins led to an attempt to increase the number of mammalian oocytes capable of fertilization and normal development. The phenomenon of superovulation was initially described by the studies of Smith and Engle (1927) and Engle (1927) in which ovulation could be induced in adult rats and mice by the daily implantation of anterior pituitary tissues. This procedure produced excessive numbers of oocytes (20-48 oocytes per animal) and implantation sites (19-29) in mice treated with mouse pituitary implants. Cole (1936) further simplified the procedure with a single injection of unfractionated pregnant mare's serum which could induce the ovulation of up to 54 oocytes. Using this simplified procedure, a maximum of 33 embryos and 23 offsprings in one litter was obtained (Cole, 1940). As regards the optimal age of experimental animals, it has been found that female rats in the period immediately before sexual maturity were more satisfactory for this purpose than adults (Cole, 1937). Under optimal conditions, 80 or more oocytes could be obtained from prepubertal mice (Gates, 1971), while 30 to 40 oocytes ovulated from mature mice (Fowler and Edwards, 1957; Biggers et al., 1971). Two possible reasons may explain the varying sensitivity to exogenous gonadotropins between prepubertal and mature animals; (1) the number of large follicles capable of responding to gonadotropins in mature mice is lower than that found in the ovaries of immature mice (Jones and Krohn, 1961), and (2) it is generally difficult to synchronize the time of administration of exogenous gonadotropins with
changes of the normal estrous cycle (McLaren and Michie, 1959; Edwards et al., 1963). Superovulation technique in sexually immature rodents appears to be more convenient, economical, and less time-consuming than spontaneous ovulation in mature donors. When a large number of pregnant animals are required for experimental purposes, we must either obtain the pregnant females from commercial distributors, or maintain enormous colonies of adult animals for mating. Both are very expensive and the latter necessitates laborious and time-consuming examination of vaginal smears. Thus, immature females in which synchronous ovulation can be induced by a single injection of PMSG may be a suitable model or substitute for adult animals in basic studies of reproduction.

It has been suggested that both the amounts of FSH and LH play a central role in ovulation and superovulation (Pincus, 1940; Evans and Simpson, 1940; Rowlands, 1944; Bodemer et al., 1959) and FSH appears to limit the ability of the follicle to respond to LH (Cole, 1936; Pincus, 1940; Willett et al., 1952; Payne and Runser, 1958). Hisaw (1947) emphasized the requirement of both adequate amount and optimum period for FSH to act before LH to ensure ovulation induction. Thus, the use of combination of PMSG, primarily a long-acting FSH-like hormone preparation, and hCG has been developed as a means of superovulation. The optimum interval between the injection of PMSG and hCG was reported to be 56 hr in the rat (Rowlands, 1944; Zarrow et al., 1958), 40 hr in the mouse (Gates and Runner, 1957; Fowler and Edwards, 1957) and 54 to 56 hr in the hamster (Bodemer et al., 1959). However, in later reports, hCG has been phased out of the two consecutive gonadotropin treatment regimens, since endogenous LH
secretion and/or exogenous LH-like activity of high dose of PMSG are thought to be sufficient to effect superovulation in females treated with PMSG alone (Whyman et al., 1979; Newcomb et al., 1979; Miller and Armstrong, 1981b). A maximum ovulatory response induced by PMSG with or without hCG has been found to be comparable in immature rats at the ages of 28 to 30 days, although the sensitivity of immature rats to a single injection of PMSG was lower than that of two consecutive gonadotropin treatments before or after these ages (Zarrow and Quinn, 1963). There is also considerable evidence that induction and synchronization of the first estrus, ovulation, mating and pregnancy can be efficiently initiated by the injection of a single dose of PMSG in immature rats (Strauss and Meyer, 1962; McCormack and Meyer, 1963; Zarrow and Quinn, 1963).

The potential of full normal development of oocytes or embryos obtained by superovulation is of obvious importance to both researchers and stock breeders. Superovulation with PMSG has been achieved in numerous species. In the rat (Cole, 1937; Sato, 1962), mouse (Fowler and Edwards, 1957; Edwards and Fowler, 1959; Wilson and Zarrow, 1962), rabbit (Kennelly and Poote, 1965; Seitz et al., 1970), hamster (Greenwald, 1962), cow (Hafez and Rajakowski, 1964; Poote and Onuma, 1970), and pig (Hunter, 1966; Dziuk, 1968; Longenecker and Day, 1968), many oocytes after superovulation could fertilize and implant to produce superpregnancy. The economic benefits of producing greater numbers of offspring from genetically superior animals has prompted the livestock industry to use this superovulation technique in attempts to obtain superpregnancy or to maximize the reproductive potential of a given female individual (Poote and Onuma, 1970; Saumande and Chupin,
1977; Baker and Jillella, 1978; Boland et al., 1978). The more recent importance in human reproduction, where superovulation using hMG equivalent to PMSG has been used for the treatment of ovarian dysfunction (Wyshak, 1978; Hack and Lunenfeld, 1979) and for IVF and embryo transfer programme (Lopata et al., 1978; Edwards, 1981; Moon et al., 1985), raises new clinical considerations.

II. Biological and Biochemical Properties of PMSG.

PMSG is one of the most widely used gonadotropin preparation to induce superovulation in various mammalian species. The simplified procedure achieved by a single dose of PMSG and its commercial availability of mass production (Papkoff, 1981) have led to its wide-scale use as a superovulatory agent in experimental and domestic animals.

PMSG has been found to be a glycoprotein hormone secreted from endometrial cups of the pregnant mare (Cole and Goss, 1943) and is present in high concentration in the circulating blood between Days 40 and 130 of gestation (Cole and Hart, 1930). Since the initial finding of the dual FSH-like and LH-like biological activities of PMSG (Cole and Hart, 1930; Cole, 1938), it has been further demonstrated that PMSG is a single molecule which consists of a hormonally non-specific subunit (PMSG-α) and a hormone-specific subunit (PMSG-β) being responsible for both FSH-like and LH-like activities (Gospodarowicz and Papkoff, 1967; Papkoff, 1974; Papkoff et al., 1978). The amino acid composition of PMSG-α beginning with the sequence NH$_2$-Phe-Pro (Gly or Pro) and terminating with isoleucine at carboxyl terminus has been found to be very similar to either LH-α, FSH-α,
or TSH-α, and the composition of PMSG-β with the sequence
NH₂-Ser-Pro-Gly.. and yet undetected C-terminal residue is closer to LH-β
(Papkoff, 1978). When comparisons have been made between PMSG and the FSH
and LH, much more carbohydrate (41-45%) has been detected in PMSG: 13.0% of
neutral sugars (mannose, galactose, and fucose), 17.6% of hexosamines
(glucosamine and galactosamine), and 10.8% of sialic acid (Landefeld, et
al., 1972; Landefeld and McShan, 1974). It has been further characterized
that PMSG-α has a 18.6% of carbohydrate content, similar to FSH-α and LH-α,
and that PMSG-β has a 55.3% of carbohydrate content which is 2.2 to 3.0 fold
higher than that of FSH-β and LH-β (Papkoff, 1978). Of its total of 55.3%
carbohydrate, sialic acid has been demonstrated to be present in the
greatest amount (21.3%) (Papkoff, 1978). In the same study, it has been
suggested that the ratio of FSH and LH activity in a PMSG molecule varies
depending upon carbohydrate composition. A radioreceptor assay utilizing a
preparation of rat testicular seminiferous tubules for measurement of FSH
concentration and rat testicular interstitial tissues for measurement of LH
levels has shown the ratio of FSH:LH in PMSG ranged from 0.87 to 1.92
depending upon the time of PMSG isolation during pregnancy and its
preparation (Stewart et al., 1976). Upon recombination of PMSG-α and PMSG-β
in a 1:1 ratio, it has been shown that significant amounts of both LH
(27.4%) and FSH (33.6%) activities were regenerated (Papkoff, 1978). The
known carbohydrate content of PMSG, specifically a very high amount of
sialic acid, accounts for the long half-life and slow clearance rate (Schams
et al., 1978).
It appears that the biochemical properties of prolonged action coupled with FSH and LH mixture may be, in part, responsible for the potency whereby superovulation can be induced by a single dose of PMSG (Zarrow and Quinn, 1963; Whyman et al., 1979; Newcomb et al., 1979; Miller and Armstrong, 1981b), since folliculogenesis in mammals depends on the presence and activities of both FSH and LH (Murphy et al., 1984). However, ovarian and superovulatory response to a standard dose of PMSG varies considerably in farm animals (Bellows and Short, 1972; Newcomb, 1976; Schams et al., 1978) and has been attributed to the possible variations in the ratio of FSH and LH activity in different extracts and commercially available batches of the hormones (Newcomb and Rowson, 1976).

III. Endocrine Aspects of Superovulation.

A. General.

The distribution and metabolism of the PMSG injected to animals have not yet been described, but a long half-life could be anticipated from the studies in various species. Endogenous PMSG in mares has a half-life of about 6 days after hysterectomy (Cole et al., 1967). Injected PMSG has a half-life of about 6 days in horses and 26 hr in rabbits (Catchpole et al., 1935), 26 hr in rats (Parlow and Ward, 1961) and about 21 hr in sheep (McIntosh et al., 1975). In sheep, McIntosh et al. (1975) found no evidence that the ovaries removed PMSG from the circulating blood; but, in cattle, low doses of PMSG injected directly into the ovary appear to bind to receptors and produce a local effect (Betteridge, 1974). The half-life of different preparations of PMSG can be expected to vary according to the
extent to which sialic acid is removed from the injected native molecule (McIntosh et al., 1975). In cycling adult hamsters treated with PMSG, Greenwald (1963) has investigated the interaction of exogenous and endogenous gonadotropins on follicular development using anti-PMSG serum and shown that the maturation of reserve follicles depends on adequate circulating levels of PMSG throughout the estrous cycle. If PMSG is required throughout the entire course of follicular development and maintenance in intact immature rats treated with PMSG, premature inactivation of PMSG may result in follicular degeneration and the loss of ovulating capability (Sasamoto and Kennan, 1972). In their subsequent study using anti-PMSG serum, it has been shown that PMSG initially stimulates follicular development during the first 36 hr following injection of the gonadotropin, and thereafter the endogenous gonadotropins elicited by PMSG via the positive feedback mechanism of steroid hormones produced are responsible for further development and maintenance of the follicles in an ovulating state (Sasamoto and Kennan, 1973). The ability of PMSG to produce superovulation appears to depend not only on its prolonged biological half-life but also on the levels being initially high enough to recruit more follicles at critical stages of their development (Greenwald, 1973).

Systemic injection of PMSG in cattle initially exerts a luteotrophic effect (Henricks et al., 1973; Hallford et al., 1975; Moore, 1975), which has been related to its LH-like activity (Newcomb and Rowson, 1976). This property has been presumed to be responsible for the occasional premature ovulation of a large follicle present at the time of PMSG injection (Newcomb and Rowson, 1976) and persistence of large nonluteinized
or cystic follicles (Betteridge, 1977). It has been suggested that the precocious formation and premature regression of CL following superovulation with PMSG inhibit further ovulation, and result in abnormal development or retardation of embryos in early pregnancy (Betteridge, 1977; du Mensil du Buisson et al., 1977). In each case, there are also suggestions that superovulatory doses of PMSG alter the ovarian steroidal milieu after ovulation and result in perturbation of oviductal and uterine function for embryo survival and/or development. Bouters et al. (1983) have reported that superovulatory treatment with PMSG in cattle induces a second wave of follicles after ovulation, causing high concentrations of 17β-estradiol in the peripheral blood during early embryonic development, which appears to be due to the long half-life of PMSG (Schams et al., 1978). Although gonadotropin treatment for multiple ovulations has frequently been reassessed (Ryan et al., 1984; Evans et al., 1984), there is little information on the synchrony of ovulation following the superovulatory treatment, despite the probability that poor synchrony is a likely cause of fertilization failure and further developmental defects of early embryos.

B. Gonadotropin Secretion and Induced Ovulation.

Spilman et al., (1973) reported that PMSG leads to the release of endogenous LH within 24 to 48 hr after injection in cattle, but this was not confirmed by others (Henricks et al., 1973; Saumande and Pelletier, 1975; Hallford et al., 1975) who describe no LH peaks before the one coinciding with estrus. Preovulatory LH secretion had no relationship with ovulation rates when expressed as maximum plasma LH levels (Henricks et al., 1973),
but a positive relationship was noticed when expressed as the total quantity of LH released (Saumande and Pelletier, 1975). In immature rats, time course studies of the ovulatory response employing defined low doses (4-8 IU) of PMSG demonstrated an integration of sequential changes in the endocrine response including gonadotropin secretion associated with a synchronized process of oocyte maturation: critical time of endogenous LH surge at 52-57 hr (Sorrentino et al., 1972; Cöstoff et al., 1974), meiotic resumption of follicular oocytes 2 to 3 hr thereafter (Hillensjo et al., 1974) and ovulation at 60-72 hr after PMSG (Walton et al., 1983). The endogenous gonadotropin surge in the PMSG-treated immature females has followed the dramatic increases in preovulatory serum levels of 17β-estradiol and progesterone which are similar to those observed during the adult proestrus (Shaikh, 1971; Butcher et al., 1974; Parker et al., 1976). The pituitary is activated to release ovulatory gonadotropins on the afternoon before estrus between 2:00 and 4:00 p.m. (known as the critical time period) which cause ovulation about 12 hrs later in cycling adult rats (Everett et al., 1949). Thus, the ovary through estrogen secretion, appears to act as a trigger for the preovulatory gonadotropin surge (Schwartz, 1969). Hillensjo et al., (1974) have reported that the temporal relationship between serum LH levels and oocyte maturation in PMSG-treated immature rats is exactly the same as that seen in proestrous adult rats.

In gonadotropin-treated immature rats, all ovulations in a particular ovary have been found to be completed in less than 1.5 hrs and frequently several follicles appear to rupture at the same time (Blandau, 1955). In the same study, the time required for a follicle to rupture has
been demonstrated to vary from 5 to 760 sec, depending largely upon the position of cumulus oophorus in relation to the stigma. However, it has been shown that high superovulatory doses of PMSG in immature rats induce precocious or multiple waves of ovulation for an unusually extended period, at least 48 hr, which are presumably due to the exogenous LH activity of PMSG in itself (De La Lastra et al., 1972; Miller and Armstrong, 1981b). Direct action of high doses of PMSG on the ovary has been demonstrated by the studies of Rowlands and Williams (1943) and Williams (1945) in which hypophysectomized immature rats ovulated after a single dose or two consecutive high doses of PMSG up to 4 days after the operation. Despite considerable attention to LH activity of PMSG and its direct action on the ovary, no published data are available on the relationship between atypical ovulatory response and amounts of exogenous or endogenous LH in the circulation of superovulated rats. Furthermore, it has been suggested that neural activation system for pituitary release of ovulating hormone may be different depending upon the doses of PMSG administered to immature rats (Ying and Meyer, 1969). In considering the dose response to PMSG, it might be relevant that, in rats, there is a depression of ovulation rate and anovulation at the doses higher than 40 IU and at the doses ranged from 12 to 15 IU, respectively (Williams, 1945; Ying and Meyer, 1969; Wilson et al., 1974). This suppression or "paradoxical" anovulatory response has been attributed to a deficiency of progesterone which synergizes with estrogen in the release of preovulatory FSH and LH (McCormack and Meyer, 1963; Carlson, 1968). In the studies above, administration of exogenous progesterone or LH prior to the critical time period overcame the inhibitory ovulatory
response. It has been reported that progesterone exhibits both facilitating and inhibiting actions on PMSG-induced ovulation in immature rats, depending upon the timing and dose of exogenous progesterone (McCormack and Meyer, 1963; Zarrow and Hurlburt, 1967). The effects of exogenous progesterone on advancement of the ovulation time may be exerted by shifting forward the critical period of endogenous LH release (Zeilmaker, 1966; Lisk, 1969). Facilitation of LH release and ovulation occurs only if progesterone is present for no more than 6 to 12 hrs; ovulation is blocked for 24 or 48 hrs if progesterone is present for a longer interval (Lisk, 1969). It has also been suggested that an adequate circulating level of estrogen is required for the spontaneous release of LH and for the facilitation of LH release by progesterone (Docke and Dorner, 1965; Grayburn and Brown-Grant, 1968).

C. Ovarian Steroids and Oocyte Maturation.

Oocytes require a specific intrafollicular steroid environment for the completion of the full maturation process. Alterations in the steroid profile during maturation, especially in preovulatory follicles, induce morphological changes in the oocyte which are expressed as gross abnormalities at fertilization including polyspermy, arrested sperm condensation and fragmented male pronuclei (Moor et al., 1980b). It has been shown that superovulatory treatment in cattle results in an abnormal progesterone/estrogen ratio in a majority of follicles during the pre- and peri-ovulatory period, and produced prematurely activated or meiotically arrested oocytes (Callesen et al., 1986). In sheep treated with superovulatory doses of PMSG, 33% of oocytes displayed a premature
activation characterized by abnormal pattern of protein synthesis and were associated with hypersecretion of follicular 17β-estradiol (Moor et al., 1984, 1985). The variation in the follicular steroidogenesis observed, during and after the preovulatory LH surge is believed to be caused first by the heterogeneity of pools of superovulated follicles which were recruited at the time of gonadotropin stimulation, and secondarily by the different ability of these follicles to develop after being stimulated (Moor et al., 1980a). Like the abnormal follicular steroidogenesis in superovulated cattle, the oocyte maturation, expressed by the nuclear and cytogenetic appearance, has been subject to a high degree of asynchrony within and between animals at the same time intervals following the LH peak (Callesen et al., 1986). This asynchrony phenomenon has been described in superovulated sheep (Moor et al., 1984) and cattle (Greve et al., 1984), and may be a consequence of premature meiotic activation of oocytes from certain follicles (Callesen et al., 1986).

Studies on sheep ovarian follicles have demonstrated that LH inhibits estrogen and androgen secretion from the Graafian follicle, while the secretion of progesterone is enhanced (Semark et al., 1974; Moor, 1974). These studies have suggested that LH has a disparate action on the steroidogenesis of preovulatory follicles: stimulation of progesterone production and inhibition of estrogen secretion. A transient stimulation of estrogen secretion seems, however, to precede the inhibition (McCracken et al., 1971). Similar evidence has been obtained by culturing preovulatory Graafian follicles, isolated from the PMSG-treated immature rats prior to the endogenous gonadotropin surge (Hillensjo et al., 1976). This study has
shown that during the first 4 hrs there is an increase in the secretion of 17β-estradiol and androgens (androstenedione and testosterone). Later, after LH treatment, there is a significant shift from estrogen/androgen to progesterone.

Several investigators have shown the effects of estrogen and progesterone on mammalian oocyte maturation in vitro. 17β-estradiol, at various concentrations, significantly reduced the incidence of maturation of cumulus-enclosed rabbit and bovine oocytes (Robertson and Baker, 1969), denuded porcine oocytes (McGaughey, 1977), and denuded mouse oocytes (Nekola and Smith, 1974; Eppig and Koide, 1978). Progesterone, at a high concentration (100 μM), has been reported to enhance the maturation of denuded bovine and rabbit oocytes (Robertson and Baker, 1969), cumulus-enclosed rabbit oocytes (Bae and Foote, 1975), and denuded oocytes from gonadotropin-treated prepubertal rhesus monkeys (Gould and Graham, 1976), while at low concentrations (10-30 μM), it decreased maturation frequency of denuded mouse oocytes (Nekola and Smith, 1974; Eppig and Koide, 1978) and corona-enclosed rabbit oocytes (Smith et al., 1978). A study on the role of hormones in gap junction function suggests that steroid hormones have a minor but statistically significant effect on the passage of molecules from cumulus cells to oocytes (Moor et al., 1980c). However, the major regulators of intracellular coupling and amino acid transport across the oolemma, namely FSH and LH, are not likely to be dependent upon steroid synthesis for their action on the membrane (Moor and Smith, 1978; Moor et al, 1980c). Therefore, it has been suggested that the action of steroid hormones is not primarily on the membranes of the oocyte (Moor et al,
1980c). Lieberman et al. (1976) have indicated that the early sequence of maturation changes in the nucleus leading to germinal vesicle breakdown and formation of the first metaphase plate are independent of steroid support. It has, however, been shown that the absence or alteration of steroid hormones during the later stages of maturation increases the incidence of chromosomal abnormality especially at telophase I and metaphase II (McGaughey, 1977; Moor et al., 1980b).

IV. Developmental Capability of Superovulated Oocytes.

A. General.

Despite widespread use of the superovulation technique in research, commercial and clinical fields, the attempts at induction of superpregnancies and excessive litter sizes in various mammalian species have been disappointing. Earlier studies found the disparity between the number of implantation sites and litter sizes (Cole, 1937; Evans and Simpson, 1940 in rats; Fowler and Edwards, 1957; Sato, 1963 in mice; Adams, 1960 in rabbits) and between ovulation and implantation rates (Austin, 1950 in rats). Results of Evans and Simpson (1940) showed that after superovulation with exogenous gonadotropins in immature rats, the maximum number of implantation sites was 34 but the maximum number of living fetuses was substantially reduced to 17. The high wastage rate has also been reported following embryo transfer in human IVF programme (Edwards, 1984; Blankstein, 1986). Despite achieving high rates of fertilization (90%) and embryo replacement (80%), pregnancy rate at term was only 15-20% per embryo transfer. In the studies above, the disparity following superovulation was
mainly ascribed to preimplantation loss of embryos, failure of implantation, or spontaneous abortion in early or mid-pregnancy.

Postimplantation resorption or death of embryos in itself may not be a major factor leading to the poor reproductive performance following superovulation. Austin (1950) was the first to focus upon preimplantation developmental failure of superovulated oocytes as a cause for the known disparity between ovulation and implantation rates and the high mating rates with few or no implantation sites in rats. Until Day 2 of pregnancy, the number of embryos flushed out of oviducts and uterine horns was comparable to ovulation counts. However, from Day 3 of pregnancy, there was a definite decline in the number of recovered embryos associated with an increase in degenerate oocytes and retarded embryo development (Wu and Meyer, 1966; Miller and Armstrong, 1981a; Sherman et al., 1982; Yun and Kwun, 1984). Early loss of preimplantation embryos following superovulation could be accounted for by the observation of vaginal expulsion of ova in both 17β-estradiol-treated rats and superovulated rabbits (Greenwald, 1961; Tsutsumi et al., 1981) and by the suggestion of excessive stimulation of the genital tract arising from hyperstimulated large follicles which persist even after normal ovulation (Miller and Armstrong, 1981b). In contrast, uterine ligation at cervical ends of superovulated rats did not increase the recovery rates in the preimplantation period (Sherman et al., 1984). Thus, expulsion of embryos to the vagina does not appear to be a main course of preimplantation loss following superovulation in rats.

There are several lines of evidence on developmental failure of superovulated oocytes. The use of exogenous gonadotropins has been linked
with detrimental effects such as chromosomal abnormalities, embryonic death and congenital defects (Bradford et al., 1974; Hofsaess and Meadham, 1971; Fujimoto et al., 1974; Takagi and Sasaki, 1976). Previous results of my M.Sc. study and others with an immature rat model have indicated that although normal pregnancy with no significant embryonic or fetal wastage can be obtained after ovulation induction with low doses of PMSG, high superovulatory doses are associated with abnormal embryo development and implantation failure (Nuti et al., 1975; Miller and Armstrong, 1981a; Sherman et al., 1982; Yun and Kwun, 1984). It has been reported that embryonic or fetal death following superovulation most commonly occurs during cleavage (Allen and McLaren, 1971), about the time of implantation and at or shortly after parturition (Edwards and Fowler, 1959; McLaren and Michie, 1959). A number of investigators have ascribed the embryo loss or death after superovulation to abnormalities or failure of the process of fertilization (Walton et al., 1983), genetic deficiencies in superovulated oocytes (Beaumont and Smith, 1975), or to environmental factors; e.g. competition between embryos (Edwards and Fowler, 1959; Edwards et al., 1963; McLaren and Michie, 1959; Allen and McLaren, 1971) or maternal systemic factors such as nutritional stress or insufficiency of progesterone (McLaren and Michie, 1956, 1959). It appears that the detrimental effects and mechanism(s) of superovulation may be multifactorial including the disturbances in oocytes/embryos themselves or in the maternal environment for their survival.
B. Genetic Condition.

Genetic abnormalities induced in oocytes/embryos might be most damaging to the usefulness of the superovulation procedure, since the progeny would be destined to death at any stage of pregnancy regardless of the normality of maternal environment (Fleming, 1982). Reports for the rabbit and mouse have indicated an increase in genetic abnormalities after superovulatory treatments compared to untreated controls (Fujimoto et al., 1974; Takagi and Sasaki, 1976) and suggested that digynic triploids constitute an appreciable proportion of pre- and post-implantation embryo losses (Takagi and Sasaki, 1976). In humans, the increased frequency of triploidy and other types of chromosomal anomalies have also been observed in spontaneous abortuses after superovulation induction therapy by hMG and hCG (Boue and Boue, 1973) and attributed to the treatment of gonadotropin itself or normal fluctuations of FSH secretion in pregnancy (Maudlin and Fraser, 1977). On the other hand, in the studies of first-cleavage mouse embryos after superovulation, a low level, statistically not significant, of chromosomal anomalies has been demonstrated: an incidence of 1.8% aneuploidy and 1.5% polyploidy (Donahue, 1972). It has also been reported that there were no increases in genetic or developmental defects in offspring after superovulation in the human (Hack and Lunenfeld, 1979), the hamster (Fleming and Yanagimachi, 1980) and the rabbit (Maurer et al., 1968). In the above studies, genetic condition of superovulated oocytes/embryos leading to embryo loss or fetal death in superovulated animals is still controversial, depending upon different species and strains, and remains to be clarified.
C. Oocyte Quality.

Normal embryo development after fertilization requires that oocytes have first attained nuclear and cytoplasmic maturity. The oocytes should be ovulated and made available for fertilization at a time coordinated with the attainment of their maturity (Fleming, 1982). The same author has suggested that oocytes released before maturity may be deficient in materials or mechanisms needed for fertilization and further development, while mature oocytes retained in follicles may suffer the loss or degeneration of these materials or mechanisms. In the pig, the proportion of primary oocytes which were ovulated into the oviduct increased with the number of total ovulations induced by increasing doses of PMSG (Hunter, 1979). Conversely, in the superovulated rhesus monkey (Batta et al., 1978), follicularly aged oocytes were obtained upon analysis of follicular oocytes at various times after ovulatory treatment, and of ovulated oocytes recovered from oviducts or uteri. In the pig, large doses of PMSG may have stimulated less-mature follicles to ovulate, whereas in the rhesus monkey, stimulation by PMSG when begun during the mid-follicular phase of the menstrual cycle may have resulted in early follicular development and enclosed oocytes to be overstimulated with respect to dose or length of exposure to FSH activity of PMSG (Fleming, 1982). It has also been suggested that late treatment of PMSG in rhesus monkeys might not "rescue" developing follicles from atresia and could lead to the ovulation of poor quality oocytes (Fleming, 1982).

Intrafollicular aging of the oocyte has been associated with an increased incidence of developmental anomalies which include an increase in polyspermy, degeneration of fertilized oocytes, irregular cell membranes,
unequal cleavage, large polar bodies, retarded development and abnormal morphology of blastocysts (Butcher and Page, 1981). A study of experimental delay of ovulation by blocking preovulatory LH surge with sodium pentobarbital in cycling adult rats has shown that preovulatory aging of oocytes in the follicles results in a decrease in fertilization rate (78% vs 93%) and an increase in abnormal development at the pronuclear, 2-cell and blastocyst stages (29.0% vs 3.6%, 14.1% vs 4.3% and 53.6% vs 20.3%, respectively), when compared to untreated controls (Fugo and Butcher, 1966). The intrafollicularly aged oocytes after a 48 hr delay of ovulation are characterized by the lack of a continuous layer of cortical granules which line the plasma membrane (Peluso and Butcher, 1974). Since the cortical layer is involved in the protective mechanism against polyspermy (Austin, 1956), the reduced cortical complement of the aged oocytes could be ineffective in blocking polyspermy, thus providing a possible explanation for the increased incidence of the developmental anomalies in the aged oocytes (Peluso and Butcher, 1974). The same authors have also suggested that premature induction of mitochondrial morphology of the follicularly aged oocytes showing elongation with a shelf-like cristae structure associated with a high metabolic state may result in the increased incidence of subsequent developmental abnormalities.

Another important source of developmental defects is anomalous fertilization caused by aging of gametes within the oviduct after ovulation (Juetten and Bavister, 1983). A number of studies have shown that delayed mating adversely affects embryogenesis (Blandau and Young, 1939; Hunter, 1980; Edwards, 1980). Rapid deterioration of the aged oocytes after
ovulation is believed to account for more than two polar bodies, formation of an extra pronucleus and decreased ability to go through early cleavage divisions (Chang and Fernandez-Cano, 1958; Juetten and Bavister, 1983). Chang (1952) inseminated rabbits at the time of induced ovulation and found that only 51% of oocytes were fertilized; about half the fertilized oocytes developed into blastocysts, and 12% of these were plainly abnormal. Even when rabbits were inseminated 2 hr before ovulation, 60% of the oocytes were lost before implantation; at a later stage, the same author noted that only 35% of implantation sites had abnormal fetuses — 6% were still developing but were abnormal, while 59% were resorbing or had already disappeared. The time relationship between oocyte aging and anomalies of fertilization has convincingly been demonstrated by Blandau (1952) who followed up his earlier studies by artificially inseminating albino rats of the Wistar strain at various times in relation to ovulation, and killing them sufficiently early to be able to observe the course of fertilization and early cleavage. The results of above study showed that, whereas only 1.4% of oocytes were undergoing abnormal fertilization when insemination was done before ovulation, there were progressively higher frequencies with the time of insemination after ovulation, rising to 14% at 6 to 8 hr and 43% at 9 to 12 hr. Degenerative changes of cumulus and corona radiata cells and other morphological changes including the loss of proximity of cortical granules to the cell membrane have been noted in mouse oocytes aged for 18 to 24 hr after ovulation (Szollosi, 1971).

In most mammals, ovulation is closely synchronized with mating, so there is little chance that, under normal circumstances, aged oocytes will
become fertilized (Juetten and Bavister, 1983). Similarly, in immature rats treated with low doses of PMSG, normal pregnancy without any significant embryonic and fetal wastage could be maintained (Nuti et al., 1975), since ovulatory response including preovulatory gonadotropin secretion and oocyte maturation is synchronized (Sorrentino et al., 1972; Costoff et al., 1974; Hillensjo et al., 1974; Walton et al., 1983). However, superovulatory doses of PMSG leads to asynchronous induction of ovulation with an extended period in immature rats (De La Lastra et al., 1972; Walton et al., 1983), in cycling adult mice (Stern and Scheutz, 1970), and in cattle (Bellows and Short, 1972). If more than one ovulation wave is occurring with superovulatory treatment, aging of the oocytes may contribute to an increased incidence of abnormal embryos (Gray and Chrisman, 1979). It appears that a pool of superovulated oocytes may include heterologous maturity. Fleming (1982) has suggested that superovulatory treatment may either "force" less-mature follicles, which lack the ability to respond fully to gonadotropic stimulation, to ovulate immature oocytes; or if initiated after the distinction of developing follicles into ovulatory and atretic subgroups may induce ovulation of over-matured or degenerating oocytes.

D. Maternal Environment.

An altered endocrine environment, especially elevated steroid concentrations, is commonly associated with superovulatory treatments in the hamster (Baranczuk and Greenwald, 1973; Greenwald, 1976), the rabbit (Fujimoto and Tanaka, 1977), the rat (Miller and Armstrong, 1981b; Walton
and Armstrong, 1981, 1982), the cow (Saumande, 1980) and the human (Vaitukaitis and Ross, 1976). Elevated steroid levels at ovulation have been suggested to exert deleterious effects on the normal development of superovulated oocytes including alteration of sperm capacitation in the female tract and oviductal transport of cleaved embryos, and interference in the formation and function of the CL (Greenwald, 1967; Chang, 1970; Humphrey, 1975).

High circulating and ovarian 17β-estradiol may be detrimental to early embryo development, since 2-cell mouse embryos cultured in oviductal fluid from estrogen-dominated donors were significantly less able to develop to morulae or blastocysts (Cline et al., 1977). Similarly, it has been shown that the influence of 17β-estradiol in the ampulla of the ovariectomized rabbit is deleterious to normal embryo development in vivo (Stone and Hamner, 1977) and in vitro (Stone et al., 1977). Greenwald (1961) found that a single injection of 17β-estradiol cyclopentylpropionate on Day 1 of pregnancy in immature rats caused the increased tubal and uterine motility so that fertilized oocytes were expelled from the uterus within 48 hr postcoitum. Thus, it has been suggested that the rapid loss of preimplantation embryos from the oviducts and uterine horns of superovulated rats may be caused by the pre- or post-ovulatory elevation of serum 17β-estradiol levels observed Days 2-5 after injection of a superovulatory dose of PMSG (Miller and Armstrong, 1981b). On the other hand, in the same study, the embryos in superovulated rats frequently remained in the oviducts until Days 6 and 7 after treatment, and their further development to morulae was impaired. The development of rat embryos in vivo to the blastocyst
stage was retarded in animals which have experienced high serum 17β-estradiol levels for an extended period prior to ovulation (Fugo and Butcher, 1966; Butcher and Pope, 1979). It has been shown that the oviducts under estrogen dominance secrete a low molecular weight substance to inhibit the development of embryos to morulae (Stone and Hamner, 1977; Stone et al., 1977; Cline et al., 1977). Therefore, there could be two possibilities of detrimental effects of high levels of circulating 17β-estradiol after superovulation on embryo development in oviducts and uteri. If transport is accelerated, embryos arriving in the uterus prematurely do not develop normally and thus may degenerate or are promptly expelled from the uterus via the cervix (Greenwald, 1961, 1967; Bennett, 1970). It has also been suggested that high levels of steroids, particularly estrogen, through the effects on oviductal musculature and sphincter, may retain the embryos in the oviduct and prevent or delay their entry into the uterus such that the synchrony between embryo and sensitized uterus is lost (Fleming, 1982).

Under normal circumstances, the secretion of steroid hormones by ovarian tissues during the estrous cycle and early pregnancy ensures that the uterine sensitivity for implantation coincides with the presence of a mature blastocyst (Psychyoyos, 1973). The same author indicated that, in the rat, both estrogen and progesterone are essential for implantation. It has been further suggested that the optimal conditions for embryo attachment and implantation depend on a delicately balanced synergistic action of estrogen and progesterone on the endometrium (Courrier, 1950), and this balance may have been disturbed by the different ratio of 17β-estradiol to progesterone in superovulated rats (Walton and Armstrong, 1982). Although, DeFeo (1963)
has shown that high levels of progesterone do not inhibit uterine sensitivity, the high levels of preovulatory progesterone in superovulated rats (Walton and Armstrong, 1981) may cause uterine changes that are detrimental to blastocyst implantation by disturbing the optimum ratio of 17β-estradiol to progesterone (Walton et al., 1982).

In addition to local or systemic endocrine changes, superovulation may alter the endocrine microenvironment through the steroidogenic capabilities of the more abundant cumulus masses and preimplantation embryos (Heap et al., 1979; Saurer, 1979; Sherizly and Kraicer, 1980).
MATERIALS AND METHODS

I. Animals.

Immature female Sprague-Dawley rats were purchased from Charles River Canada Inc. (St. Constant, Quebec) at 22 days of age. All animals were kept under temperature- and light-controlled conditions (20-25°C, 12L:12D) in an air-conditioned room and fed standard rat chow and water ad libitum throughout the treatment period.

II. Experimental Design.

Protocol 1. The effects of different doses of PMSG on ovulatory response and oocyte quality (gross morphology) were examined with reference to steroid hormone levels in ovarian tissues and peripheral blood. At the age of 28 days, animals received 4 IU (control dose), 20 IU (intermediate superovulation dose), or 40 IU (high superovulation dose) of PMSG between 0830 and 0900 h. PMSG (Equinex, Ayerst, Montreal, Quebec) was subcutaneously administered in 0.4 ml of 0.9% NaCl solution. Following PMSG injection, the rats were killed by cervical dislocation at 6 or 12 hr intervals (n=5-8 rats at each time point).

Trunk blood was collected in glass tubes and allowed to stand at room temperature for 1/2-1 hr to clot. The samples were then stored for 12-24 hr at 4°C and centrifuged at 2000 rpm for 10 min to separate serum. At the time of sacrifice, ovaries were dissected free from oviducts, cleaned of bursae, connective tissue and fat, and crushed in 1 ml absolute ethanol with a glass rod. The sera and ovarian homogenate were stored at -20°C.
for subsequent assay of 17β-estradiol, progesterone and androgens using RIA. Some ovaries obtained at 24 or 48 hr after PMSG injection were processed for histological examination of follicles.

Oocytes were collected from oviducts and immediately subjected to the assessment of ovulatory response and oocyte gross morphology.

Protocol 2. To examine the follicular steroid contents and oocyte maturation following PMSG treatments, animals were allotted to the two groups receiving 4 IU or 20 IU of PMSG at the age of 28 days, and killed at 24, 48, 60 or 72 hr after PMSG (n=5-7 rats at each time point).

Ovaries obtained at each time period were dissected free, cleaned of bursae, connective tissue, and fat. A heat-drawn micropipette connected to 20 cm of plastic tubing (i.d., 1 mm) was attached to a 10 ml syringe. Under a dissecting microscope (Nikon, Optiphot), each follicle was carefully punctured and follicular fluid was collected by suction. The follicular fluid pooled from all visible antral follicles on each pair of ovaries was expelled into a narrow centrifuge tube. After centrifugation at 1000 rpm for 5 min, the volume of supernatant follicular fluid was measured with an Eppendorf mechanical pipette (0.5-10 µl). The volumes of follicular fluid obtained from each pair of ovaries ranged from 2-10 µl; they were made up to a total volume of 1.0 ml with saline and stored at -20°C for the subsequent assay of 17β-estradiol, progesterone, and androgens.

The oocytes, recovered from oviducts at 72 hr (4 IU PMSG) and 24, 48 or 72 hr (20 IU PMSG), were classified as degenerate or normal in appearance. Only morphologically normal oocytes were subjected to the staining of nucleus for the assessment of each stage of nuclear maturation.
Protocol 3. To examine the circulating LH response to PMSG treatments, animals were divided into the two groups receiving 4 IU or 20 IU of PMSG (n=7 samples at each time point).

One day before the experiment (Day 27 of age), the rats were installed with chronically indwelling catheters using the method described by Harms and Ojeda (1974). Briefly, the right jugular vein was exposed after induction of anesthesia with sodium pentobarbital (Somnotal, Sigma Chemical Co., St. Louis, MO) at a dose of 35 mg/kg of body weight and a curved 22-gauge needle was used to place a catheter made of silastic tubing (Dow-Corning Corp., Midland, MI) into the vein. The catheter was slid into the vein and made to enter or approach the right atrium. The catheter was then anchored onto the pectoral muscle under which the jugular vein passes. The exposed end of the catheter was then passed underneath the skin and exteriorized at the back of the neck and tied shut.

On the following day, the jugular catheter was connected with a flexible piece of heparinized polyethylene tubing (Fisher Scientific Co., NJ, PE-50) for withdrawal of blood samples. To provide the basal level of serum LH, 0.5 ml of whole blood was collected from individual rats via the jugular catheter immediately before administration of PMSG. All rats in both groups were bled 0.5 ml whole blood at 12 hr and subsequently at 6 hr intervals until sacrifice at 72 hr after PMSG. To prevent acute shock from blood loss and clot formation in the catheter, each blood sample was followed by replacement of an an equal volume of a dilute heparin-saline solution (25 IU/ml) as described elsewhere (Waynforth, 1980). Serum samples (about 200 µl per tube) were separated by centrifugation and stored at -20°C until LH RIA.
At the time of sacrifice at 72 hr, ovaries were dissected and weighed as a pair. Ovulation was also assessed by counting the oocytes recovered from oviducts.

Protocol 4. The effects of an androgen antagonist (flutamide) on oocyte quality (gross morphology) and developmental potential of preimplantation embryos after treatments with PMSG were examined. Initially, animals were divided into the two treatment groups receiving 5 mg flutamide or ethanol-saline vehicle at 30 and 36 hr, and one pre-treatment control group receiving no further treatment after administration of 40 IU PMSG at the age of 28 days. The animals of treatment groups were killed at 48, 60 or 72 hr after PMSG (n=6 or 7 rats at each time point) and those of pre-treatment control group were killed at 24 hr (n=6 rats). Flutamide (α,α,α-trifluoro-2-methyl- 4'-nitro-m-lacto-toluidide, Schering Pharmaceuticals, Kenilworth, NJ) was dissolved in ethanol and then diluted with an equal volume of saline as described previously (Peluso et al., 1981), and subcutaneously administered in a volume of 0.4 ml.

In an extension to the initial experiment, the rats subsequently to be mated, were randomly allotted to the three different groups. The females of one group received 4 IU PMSG alone. The females of the other two groups were given 40 IU PMSG with either flutamide or ethanol-saline vehicle as described previously. At 60 hr after PMSG, the females had their vaginae gently opened with saline-soaked cotton swabs and were caged with mature fertile Sprague-Dawley males (one male and two females per breeding cage). The females were separated from males and scored for the occurrence of mating at 72 hr (designated as Day 1 of pregnancy). A sperm-positive score
was noted by the presence of a copulatory plug in the vagina and/or spermatozoa in vaginal smears. The mated females were killed between 0900-1000 h on Days 2, 3, 4 or 5 of pregnancy (n=5 or 6 rats on each day of pregnancy).

At the time of sacrifice in both the experiments, ovarian tissues and/or blood were prepared and stored for the assay of 17β-estradiol, progesterone, and androgens. Oocytes and embryos were collected from oviducts and/or uterine horns, and subjected to the assessment of ovulation, gross morphology of oocyte or preimplantation embryo, and developmental stage.

Another separate experiment was performed to determine major androgenic steroids in serum of PMSG-and flutamide-treated rats. The rats received 4 IU PMSG alone or 20 IU PMSG with either a combination of flutamide or ethanol-saline vehicle, and their trunk blood samples were collected at 24, 48, or 60 hr (n=10 samples at each time point). The dose and time schedule for the treatment of flutamide was identical to the precedings. The sera were separated by centrifugation and stored at -20°C until the subsequent separation of androgenic steroids using TLC. The levels of each steroid were determined using RIA.

Protocol 5. The effects of a progesterone antagonist (RU486) and an estrogen antagonist (tamoxifen) on ovulatory response and oocyte quality (gross morphology) after treatments with PMSG were examined. Initially, animals received 4 IU PMSG at the age of 28 days and were divided into three groups receiving three consecutive injections of 1 mg RU486, 1 mg tamoxifen or vehicle alone at 24, 36 and 48 hr after PMSG. All rats were sacrificed
at 72 hr (n=7 rats). RU486 (RU38486, 11β-(4-dimethylaminophenyl)-17β-hydroxy, 17α-(prop-1-ynyl)-estra-4,9-dien-3-one, Roussel Uclaf, Romainville, France) and tamoxifen (I.C.I. 46,474, trans isomer of 1-(p-β-dimethylaminoethoxyphenyl) -1,2-diphenylbut-1-ene, Sigma Chemical Co., St. Louis, MO; free base) were subcutaneously administered in 0.4 ml of sesame oil suspension containing 5% ethanol as a vehicle.

In an extension to the initial experiment, the rats were injected with 40 IU PMSG and divided into four groups receiving either no further injections (pre-treatment control), 1 mg RU 486, 2.5 mg tamoxifen, or vehicle alone following the injection schedule described previously. The females were killed at 12 hr (pre-treatment control), 60 or 72 hr after PMSG (n=5-7 rats at each time point).

At each time of sacrifice, trunk blood was collected and stored at 4°C prior to the separation of serum by centrifugation for the assay of 17β-estradiol, progesterone, and androgens. Ovaries were dissected and weighed as a pair. Oocytes were recovered from oviducts and subjected to the assessment of ovulation and oocyte gross morphology.

III. Assessment of Ovulation, Oocyte Morphology and Embryo Development.

Oviducts and uterine horns were separated at the uterotubal junction. The oviducts obtained from each female were placed in a few drops of DPBS in a 10x35 mm petri dish (Falcon Plastics, Los Angeles, CA). Under a stereo dissecting microscope (10X magnification, Nikon SMZ-10), oocyte mass was readily accessible in the enlarged and translucent ampulla region of the oviduct. This distended segment was then punctured and the oocyte
mass was expelled. Subsequently, in order to facilitate the oocyte counting, the extracoronal cumulus cells surrounding the oocytes were dispersed after being exposed to a few drops of DPBS containing 0.1% of hyaluronidase (ovine type II, Sigma Chemical Co., St. Louis, MO) for 5-10 min.

Specifically, in pregnant females of the protocol 4, the oviduct was flushed with 0.2 ml DPBS by inserting a blunt-ended 30 gauge needle through the infundibulum, and the uterine horn was flushed with 0.5 ml DPBS by inserting a blunt-ended 21 gauge needle through the wall of its cervical end.

The recovered oocytes or embryos were counted under the stereo dissecting microscope (40X magnification) and examined without staining under a phase-contrast microscope (100X magnification, Nikon Optiphot). The occurrence of fragmentation and other degenerative changes was assessed, as described elsewhere (Elsden et al., 1978; Miller and Armstrong, 1981b). Briefly, those eggs showing an irregular cell mass with debris, an armophous opaque mass of vitelline material, empty zona pellucida, a great difference in size of blastomeres and a hazy blastomere outline were classified as abnormal. A major proportion of abnormalities was derived from fragmentation. The developmental stages of preimplantation embryos were also recorded.

IV. Assessment of Oocyte Nuclear Maturation.

Normal appearing oocytes were placed into the Falcon petri dish containing hypotonic sodium citrate (1.0%, Anala R, Toronto) and swollen at
room temperature for about 10 min. The oocytes were subsequently transferred onto a grease-free slide with a thin coat of Mayer's albumen (Jones, 1966), a mixture of an equal volume of chicken egg white and glycerol (BDH Chemicals Ltd., Toronto, Ontario), and allowed to dry over a hot plate (45-53°C) to enhance chromosome spreading. Mayer's albumen was used to attain better adhesion of the oocytes onto the slide, thus preventing the loss in the next step of fixation. The dried oocytes were then fixed with acetic alcohol, freshly made up with one part of glacial acetic acid (BDH Chemicals Ltd., Toronto, Ontario) and two parts of absolute ethyl alcohol (BDH Chemicals Ltd., Toronto, Ontario), in a Coplin jar for 45 min and stained with 2% aceto-orcein for 30 min. A stock solution of this stain had been made up in a fumehood by dissolving 2.2 g of orcein (B34063, BDH Chemicals Ltd., Toronto, Ontario) in 45 ml of hot glacial acetic acid with continuous stirring for 30 min and by adding 55 ml of 0.9% NaCl solution (Tjio and Whang, 1962). A working staining solution was prepared immediately before use by mixing two parts of the stock solution with three parts of 0.9% NaCl solution and by filtering with Millex PF filter (Millipore Products Division, Bedford, MA). Finally, the stained oocytes were allowed to a serial dehydration through 50%, 60%, 80% and 100% ethanol followed by xylene (Anala R, Toronto, Ontario) for 5 min in each step, and subjected to a microscopic evaluation of nuclear maturation. Various stages of meiosis observed in this experiment were identified according to the criteria of Austin (1961, see Figure 1).
FIGURE 1

Diagrammatic representation of preovulatory nuclear maturation stages in the rat oocyte (re-drawn from Austin, 1961).

a: 'Germinal vesicle' stage

b: 'Germinal vesicle' break-down (GVBD) stage, migration of germinal vesicle to the periphery of ooplasm, and disappearance of nucleolus and nuclear membrane

c: Prophase I, speckled appearance with short segments of chromosomal threads.

d: Metaphase I, arrangement of the bivalents at the equator of meiotic spindle.

e: Anaphase I, movement of the bivalents to the opposite ends of the spindle.

f,g: Telophase I, rotation of the spindle through 90°, and start of polar body separation with elevation of vitellus near to the chromosomes.

h,i: Metaphase II, emission of the first polar body with formation of a furrow around the outermost set of chromosomes.
V. Histological Examination.

Ovaries obtained at 24 or 48 hr after 4, 20 or 40 IU PMSG were immediately fixed in Bouin's solution (saturated picric acid - 75%, buffered formalin - 20%, glacial acetic acid - 5%, all reagents obtained from BDH Chemicals Ltd., Toronto, Ontario) for about 4 hr and washed for 2 to 3 hrs in running tap water to remove excess fixative. The ovarian tissue was subsequently dehydrated in sequential concentrations of ethanol (70, 80, 90 and 100%), cleared, and embedded in paraffin wax. Serial sections of the tissue block were then cut 10 µm thick and stained with hematoxylin (CIN 75290, BDH Chemicals Ltd., Toronto, Ontario) and eosin (CIN 45380, BDH Chemicals Ltd., Toronto, Ontario). All sections were examined for evidence of precocious ovulation or follicular atresia, and representative sections were taken for photomicroscopy. Advanced stages of follicular atresia were defined by nuclear pyknosis and/or a loss of homogeneity of the granulosa cells in conjunction with thecal hypertrophy and varying degrees of oocyte degeneration.

VI. Determination of Hormones.

A. 17β-estradiol, Progesterone and Androgens.

Ovarian supernatants obtained by centrifugation, 0.5-1.0 ml aliquots of sera and follicular fluid samples were extracted twice with 5.0 ml diethyl ether (anhydrous, BDH Chemicals Ltd., Toronto, Ontario) by vortexing for 2 min; the extracts were evaporated under nitrogen flow at 35°C and reconstituted in 1.0 ml absolute ethanol. A range of 20-100 µl aliquots of the extracts, if necessary, after 10 or 100 times of dilution
with absolute ethanol, were dried down and assayed in duplicate for 17β-estradiol, progesterone and androgens by specific RIA using the antisera kindly donated by Dr. D.T. Armstrong, University of Western Ontario, London, Ontario. Non-radiolabelled steroids, 17β-estradiol, progesterone and testosterone purchased from Sigma Chemical Co. (St. Louis, MO), and radiolabelled steroids [2,4,6,7,16,17-\(^3\)H]17β-estradiol (sp. act. 140 Ci/mmol), [1,2,6,7,16,17-\(^3\)H]progesterone (sp. act. 112 Ci/mmol) and [2,6,7-\(^3\)H]testosterone (sp. act. 80 Ci/mmol) obtained from Amersham Co. (Arlington Heights, IL) were used as standard and tracer, respectively. Solvents obtained for steroid RIA were of analytical grade and used without further purification, except for ethanol, which was redistilled.

In the assay procedures, approximately 10,000 cpm of tracer (\(^3\)H) was added to each tube. The unbound hormone was removed by the dextran-coated charcoal adsorption method, and the bound steroid was counted in a LKB 1217 Rackbeta liquid scintillation counter. The assays were linear at 25-300 pg/tube. The binding efficiency (Bo) of the steroid antibodies was 40-60% and NSB was less than 5%. The intra- and inter-assay CV for the steroid assays were as follows: 7% and 5% for 17β-estradiol, 8% and 9% for progesterone, 5% and 6% for androgens, respectively. The respective cross-reactivities of the antisera were as follows:
<table>
<thead>
<tr>
<th>Steroid Antibody</th>
<th>Compound</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>17β-estradiol Antibody</strong></td>
<td>17β-estradiol</td>
<td>100.0%</td>
</tr>
<tr>
<td></td>
<td>estradiol</td>
<td>100.0%</td>
</tr>
<tr>
<td></td>
<td>estradiol</td>
<td>2.9%</td>
</tr>
<tr>
<td></td>
<td>estriol</td>
<td>0.5%</td>
</tr>
<tr>
<td></td>
<td>other major steroids</td>
<td>&lt;0.2%</td>
</tr>
<tr>
<td><strong>Progesterone Antibody</strong></td>
<td>progesterone</td>
<td>100.0%</td>
</tr>
<tr>
<td></td>
<td>5α-pregnane-3,20-dione</td>
<td>35.5%</td>
</tr>
<tr>
<td></td>
<td>5α-pregnane-3,20-dione</td>
<td>15.7%</td>
</tr>
<tr>
<td></td>
<td>3α-hydroxy-5β-pregnan-20-one</td>
<td>2.0%</td>
</tr>
<tr>
<td></td>
<td>20β-hydroxy-4-pregnene-3-one</td>
<td>1.3%</td>
</tr>
<tr>
<td></td>
<td>17α-hydroxyprogesterone</td>
<td>1.2%</td>
</tr>
<tr>
<td></td>
<td>other major steroids</td>
<td>&lt;0.2%</td>
</tr>
<tr>
<td><strong>Testosterone Antibody</strong></td>
<td>testosterone</td>
<td>100.0%</td>
</tr>
<tr>
<td></td>
<td>5α-dihydrotestosterone</td>
<td>75.0%</td>
</tr>
<tr>
<td></td>
<td>5α-androstane-3α,17β-diol</td>
<td>13.5%</td>
</tr>
<tr>
<td></td>
<td>5α-androstane-3β,17β-diol</td>
<td>10.9%</td>
</tr>
<tr>
<td></td>
<td>19-hydroxytestosterone</td>
<td>4.7%</td>
</tr>
<tr>
<td></td>
<td>other major steroids</td>
<td>&lt;0.1%</td>
</tr>
</tbody>
</table>

Based on the above cross-reactivity data, the testosterone antibody was relatively nonspecific, thus the steroids measured using this antiserum were referred to as androgens rather than testosterone. Hormone levels were
expressed as ng/rat for ovarian steroids, and ng/ml for serum and follicular steroids.

B. Androgenic Steroids.

The separation of major androgenic steroids (androstenedione, testosterone, and 5α-dihydrotestosterone) from sera was undertaken by TLC. The 1.0 ml aliquots of sera were extracted twice with five volumes of diethyl ether by vortexing for 2 min; the extracts were evaporated under nitrogen flow at 35°C. Approximately 4000 dpm of [4-¹⁴C] androstenedione (sp. act. 59.0 mCi/mmol), [4-¹⁴C] 5α-dihydrotestosterone (sp. act. 59.0 mCi/mmol), and [4-¹⁴C]testosterone (sp. act. 56.9 mCi/mmol) obtained from Amersham Co. (Arlington Heights, IL) were added as internal standards to the dried extracts and reconstituted in 70 µl of absolute ethanol. The extracts were applied onto silica-coated TLC plates (Eastman Kodak Co., Rochester, NY), and the androgenic steroids were separated in a system of chloroform:methylene chloride:diethyl ether (40:40:20,v:v:v) at 4°C. These solvents, obtained from BDH Chemicals Ltd. (Toronto, Ontario), were of analytical grade and used without further purification. The isolated steroids were detected by exposing the chromatograms to no-screen medical x-ray film (Eastman Kodak Co., Rochester, NY) for 7 days. Later, the standard steroids were visualized by spraying the chromatograms with sulfuric acid (J.T. Baker Chemical Co., Phillipsburg, NJ):ethanol (1:1,v:v) and by charring for 15 min at 120°C in Fisher 30 G Lab. oven (see Figure 2). The steroid spots on the chromatograms were initially identified by aligning and transilluminating with the corresponding
FIGURE 2

Separated steroids on TLC silica gel-coated plate, developed in a solvent system of chloroform:methylene chloride:diethyl ether (40:40:20, v:v:v). The developed chromatograms were exposed to no-screen x-ray film in order to localize labelled androgenic steroids.
PROGESTERONE
ANDROSTENEDIONE
5α-DIHYDROTESTOSTERONE
17β-ESTRADIOL
TESTOSTERONE
ORIGIN
autoradiograms. Final identification of each steroid was made by recrystallization to a constant specific activity (see Table I), which was carried out on noncharred steroids eluted from the appropriate zones of the chromatograms. The spots were cut out and stored in ether-rinsed culture tubes. The androgenic steroids were eluted from the cut-out spots by using twice 5.0 ml of diethyl ether. After evaporation, the final extracts were reconstituted in 1.0 ml of absolute ethanol and stored at -20°C prior to steroid RIA.

To determine the procedural loss for TLC separation of androgenic steroids, steroid free serum was used. Normal rat serum obtained from immature rats without any treatment was mixed with activated Norit-A charcoal (Fisher Scientific Co., NJ) at a ratio of 2 g in 10 ml serum and then incubated for 24 hr at 4°C. The resulting slurry was centrifuged four times at 4000 rpm for 30 min. This procedure was reported to remove over 99% of steroids from serum without affecting total protein concentration or pH (Hollander and Shenkman, 1974). Subsequently, known quantities of all the three radiolabelled androgenic steroids were added to the steroid-free serum and separated by TLC as described previously. The procedural loss for TLC separation was less than 30%; recovery rates for the three androgenic steroids were the followings: androstenedione, 81%; 5α-dihydrotestosterone, 71%; testosterone, 82%.

The androgenic steroids separated from serum samples were determined by the same steroid RIA procedure described previously. In the assay procedure, since testosterone antiserum showed a substantially high cross-reactivity to 5α-dihydrotestosterone, the contents of the two
TABLE I

Radiochemical identification of androstenedione, 5α-dihydrotestosterone and testosterone*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Carrier</th>
<th>Specific activity (dpm/mg) after recrystallization number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg)</td>
<td>0</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>11.7</td>
<td>391</td>
</tr>
<tr>
<td>5α-dihydrotestosterone</td>
<td>12.4</td>
<td>495</td>
</tr>
<tr>
<td>Testosterone</td>
<td>11.3</td>
<td>362</td>
</tr>
</tbody>
</table>

* Recrystallizations were carried out from acetone and hexane.
steroids, testosterone and 5α-dihydrotestosterone were assayed with testosterone antiserum. However, the measurement of androstenedione contents was made with its specific antiserum kindly donated by Dr. D.T. Armstrong. In this assay, non-radiolabelled androstenedione obtained from Sigma Chemical Co. (St. Louis, MO) and radiolabelled steroid [1,2,6,7-^3H]-androstenedione (sp. act. 110 Ci/mmol) purchased from Amersham Co. (Arlington Heights, IL) were used as standard and tracer, respectively. Hormone levels were expressed as ng/ml for serum androgenic steroids.

C. LH.

The serum samples collected at 6 or 12 hr intervals for 72 hr after administration of 4 or 20 IU PMSG were analyzed in duplicate by a standard LH assay procedure with a slight modification of a double-antibody RIA method as described by Murphy and Mahesh (1984). The purified LH antigen (NIH-rLH-I-6), standard (NIH-rLH-RP-2) and the first antibody (NIH-anti-rLH-S-8) were obtained from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases (NIADDK) National Hormone and Pituitary Program. The purified LH antigen was iodinated with $^{125}$I by the Chloramine-T method (Bolton, 1977). The second antibody (sheep anti-rabbit gamma globulin) was purchased from Arnell Products Co., Inc. (Cherokee Station, New York, NY). A 25% binding was obtained at the final dilution of 1:180,000. The assay was linear at 80-640 pg/tube. The volume of the serum aliquots (100 μl) was adjusted to work in the middle range of the standard curve. All samples were measured in a single assay, in which a pool of serum from intact cycling adult rats on the day of diestrus had a
level of 0.98 ng LH/ml with the intra-assay CV of 6.4%. The minimum
detectable level of LH was 40 pg/tube, or 0.4 ng/ml in the samples. The
values of serum LH were expressed in terms of ng NIH-rLH-RP-2/ml.

In the assay procedure, PMSG was found to cross-react with the LH
antiserum, although the binding was not parallel to that of NIH-LH-RP-2 (see
Figure 18). The addition of 0.125 IU PMSG to 100 µl serum from the intact
rats raised the apparent level of "LH" in the assay by about 300 pg
NIH-LH-RP2 equivalent. This was approximately equal to the increase in
serum immunoreactive LH that was measured in the rats of superovulation
group following the injection of 20 IU PMSG (see Figure 17).

VII. **Statistical Analysis**

Data generated from the experiment of Protocol 1 were evaluated by
a completely randomized design of analysis of variance (Steel and Torrie,
1960). When significant interactions were found, individual F-tests were
performed to determine the significance of differences between any means of
groups. Differences between means for all the data were considered to be
significant at p<0.01. Correlation between ovarian or serum steroid levels
and percentages of abnormal oocytes was also assessed using the Spearman's
rank correlation test.

Data from the experiments of Protocols 2 to 5 were analyzed
employing Student's t-test, or when appropriate, analysis of variance
followed by Fisher's PLSD test with the aid of a computerized program.
Comparisons with p<0.05 were considered to be significant.
RESULTS

I. Oocyte Morphology, Ovulatory and Steroid Responses (Protocol 1).

The time course of PMSG-induced ovulatory response is presented in Figures 3 and 4. All control rats (4 IU PMSG) ovulated at 60-72 hr, while the rats given superovulatory doses (20 and 40 IU) of PMSG began ovulating as early as 24 hr after gonadotropin injection. Both superovulatory treatments yielded a comparable percentage (28.6%) of ovulating animals at 24 hr after injection. Thereafter, this effect was dose-dependent; 20 and 40 IU PMSG induced ovulation in over 60% of the animals at 48 and 36 hr, respectively. There were two increases in the number of oocytes recovered from oviducts of the superovulated rats: a dose-dependent increase between 24 and 36 hr and a dose-independent increase between 48 and 72 hr. After 48 hr, the superovulated animals showed a significant (p<0.01) increase in the number of released oocytes above that of 4 IU PMSG-treated rats. The maximum counts of the oocytes were attained for 20 IU PMSG treatment at 60 hr (57.6±2.9 oocytes/rat) and for 40 IU PMSG at 72 hr (50.5±5.0 oocytes/rat).

Abnormal oocytes with various types of degeneration were recovered from 48 and 36 hr onwards in the rats given 20 and 40 IU PMSG, respectively (Fig. 5). In contrast, no degenerate oocytes were found in any control rats throughout the whole examination period. The percentage of abnormal oocytes generally increased in a dose-dependent manner in the superovulated rats given both 20 and 40 IU PMSG (Fig. 6). Sharp increases in the percentage of abnormal oocytes occurred from 54 hr onwards for 20 IU PMSG-treated rats and at 36-48 hr and 54-60 hr for 40 IU PMSG-treated rats. The maximum degeneration of oocytes was observed in 20 IU PMSG-treated rats at 72 hr.
FIGURE 3

Percentage of rats ovulated at different time periods after injection of 4, 20 or 40 IU PMSG. Numbers above each bar represent the number of rats sacrificed at each time; ~ denotes 0%.
FIGURE 4

Recovery of oocytes from oviducts per rat at different time periods after injection of 4, 20 or 40 IU PMSG. Values represent means ± SEM (n=5-8).
FIGURE 5

Morphology of normal and abnormal oocytes recovered from oviducts after treatments with a control dose (4 IU) and superovulatory doses (20 and 40 IU) of PMSG, respectively.

a: Normal appearing oocyte with the first polar body (PB) at 72 hr (4 IU PMSG), x400.

b-d: Abnormal oocytes with different sizes of ooplasmic fragments at 48-72 hr (20 IU PMSG), x400.

e: Abnormal oocyte with contracted ooplasm at 72 hr (40 IU PMSG), x400. A part of ooplasm is pulled away from the fractured zona pellucida.

f: Abnormal oocyte with "empty zona" structure at 72 hr (40 IU PMSG), x400.
FIGURE 6

Percentage of abnormal oocytes recovered from oviducts of superovulated rats at different time periods after injection of 20 or 40 IU PMSG. Numbers above each bar represent the total number of oocytes recovered at each time; ~ denotes 0%.
(30.9%) and in 40 IU PMSG-treated rats at 60 hr (61.0%). The majority of oocytes undergoing degeneration was free of cumulus masses. Histological examination of ovaries from the superovulated rats showed freshly ruptured and luteinized follicles indicating precocious ovulation as early as 24 hr after PMSG treatment (Fig. 7). In contrast, the ovaries of 4 IU PMSG-treated rats showed no evidence of ovulation up to 48 hr. In large follicles of the control rats, there was little evidence of follicular atresia with oocyte degeneration at 24 or 48 hr after injection (Fig. 8). However, in large follicles of the superovulated rats, a predominance of follicular atresia with fragmentation of oocytes was observed at 48, but not at 24 hr, after treatment (Fig. 9).

Ovarian contents of 17β-estradiol, progesterone and androgens are shown in Figure 10. At 0 hr, the ovaries contained 0.2±0.0 ng 17β-estradiol/rat, 1.9±0.6 ng progesterone/rat and 0.5±0.0 ng androgens/rat. Following PMSG injection, the 17β-estradiol content of ovaries from control rats (4 IU PMSG) rose gradually to a peak of 4.6±0.5 ng/rat at 48 hr (proestrus) and then sharply declined to less than 0.7 ng/rat at 60 and 72 hr after injection. Compared to controls, the 17β-estradiol content of superovulated ovaries increased significantly (p<0.01) between 36 and 60 hr. The peak values of 17β-estradiol were 18.4±3.2 ng/rat and 9.1±0.5 ng/rat for 20 IU PMSG (at 60 hr) and for 40 IU PMSG (at 54 hr), respectively. By 72 hr, ovarian 17β-estradiol content was comparable in 4 IU (0.2±0.0 ng/rat), 20 IU (0.6±0.0 ng/rat) and 40 IU (1.3±0.2 ng/rat) PMSG-treated rats. Ovarian progesterone content in 20 IU PMSG-treated rats increased steadily from 12 hr onwards and reached a peak
FIGURE 7

Ovarian histology obtained at 24 hr after 20 IU PMSG treatment. Note a ruptured follicle with abundant luteal cells in the antrum. a) x100, b) x1000.
FIGURE 8

Follicular and oocyte morphology of control ovary obtained at 48 hr after 4 IU PMSG treatment. Note appearance of healthy large follicle with a normal oocyte. a) x100, b) x1000.
FIGURE 9

Follicular and oocyte morphology of superovulated ovary obtained at 48 hr after 40 IU PMSG treatment. Note atretic large follicle with a fragmented oocyte; there are loss of granulosa cell continuity (1) and hypertrophy of theca layer (2). a) x100, b) x1000.
FIGURE 10

Changes in ovarian 17β-estradiol, progesterone and androgens after administration of 4, 20 or 40 IU PMSG to immature rats. Values represent means ± SEM for paired ovaries (n=5-8).
at 60 hr (133.0±18.8 ng/rat), whereas in the rats given 40 IU PMSG, it showed a biphasic increase with the peak values at 36 hr (196.3±12.1 ng/rat) and at 60 hr (205.1±17.6 ng/rat). In 4 IU PMSG-treated rats, there was no significant increase in the progesterone content during the first 54 hr, but thereafter it increased sharply to a peak at 60 hr (127.0±9.1 ng/rat). By 72 hr, ovarian progesterone content was 72.5±17.3 ng/rat for 4 IU PMSG-treated rats. It was, however, significantly (p<0.01) higher in the superovulated rats: 130.4±23.4 ng/rat (20 IU) and 162.2±6.6 ng/rat (40 IU). Ovarian androgen content in the control rats given 4 IU PMSG demonstrated two peaks: 50.9±6.0 ng/rat at 12 hr and 52.9±8.0 ng/rat at 36 hr after injection. It was significantly (p<0.01) higher between 12 and 36 hr, when compared to the superovulatory treatment of 20 or 40 IU PMSG. From then onwards, the ovarian androgen content for 4 IU PMSG-treated rats sharply declined to less than 5.7 ng/rat at 60 or 72 hr. In contrast, androgen content of the superovulated ovaries increased and between 48 and 60 hr was significantly (p<0.01) higher than that of control ovaries, with the peak values of 58.6±7.7 ng/rat at 60 hr after treatment with 20 IU PMSG and 61.2±5.1 ng/rat at 54 hr after administration of 40 IU PMSG. By 72 hr, ovarian androgen content was comparable in 4 IU (2.5±0.8 ng/rat) and 20 IU (2.9±0.6 ng/rat) but higher in 40 IU (14.3±2.6 ng/rat) PMSG-treated rats. The changes in the 17β-estradiol, progesterone and androgens of ovarian tissues and sera followed comparable profiles throughout the whole examination period (Figs. 10 and 11). The peak levels of ovarian steroids were, however, much higher than those of serum steroids: up to 4-fold for 17β-estradiol, up to 2-fold for progesterone and up to 7-fold for androgens.
FIGURE 11

Changes in serum 17β-estradiol, progesterone and androgens after administration of 4, 20 or 40 IU PMSG to immature rats. Values represent means ± SEM (n=5-8).
17β- Estradiol

- 4IU PMSG
- 20IU PMSG
- 40IU PMSG

Serum Steroids (ng/ml)

Progesterone

Androgens

Time (hour)
There was a significant positive correlation between ovarian or serum androgen levels and percentages of abnormal oocytes during the interval of androgen increase, i.e. between 24 and 60 hr after injection of 20 or 40 IU PMSG (Figs. 12 and 13). Regression analysis gives the equations: for 20 IU PMSG treatment, \( y=0.23x_1-2.49 \) (\( r=0.81, p<0.01, n=16 \)) and \( y=1.07x_2-2.92 \) (\( r=0.85, p<0.01, n=16 \)); for 40 IU PMSG treatment, \( y=0.70x_1+1.58 \) (\( r=0.51, p<0.05, n=25 \)) and \( y=4.05x_2-5.07 \) (\( r=0.70, p<0.01, n=25 \)), where \( y= \) percentage of abnormal oocytes, \( x_1= \) content of ovarian androgens, and \( x_2= \) concentration of serum androgens.
FIGURE 12

Correlation between the percentage of abnormal oocytes and content of ovarian androgens from 24 to 60 hr after injection of 20 or 40 IU PMSG to immature rats.
53

Ovarian Androgens (ng/rat)

% Abnormal Oocytes

40 IU PMSG:
\[ r = 0.51, y = 0.70x + 1.58 \]

20 IU PMSG:
\[ r = 0.81, y = 0.23x - 2.49 \]
Correlation between the percentage of abnormal oocytes and concentration of serum androgens from 24 to 60 hr after injection of 20 or 40 IU PMSG to immature rats.
Serum Androgens (ng/ml)

% Abnormal Oocytes

40 IU PMSG:
\[ r = 0.70, \quad y = 4.05x + 5.07 \]

20 IU PMSG:
\[ r = 0.85, \quad y = 1.07x + 2.92 \]
II. Follicular Steroid Contents and Oocyte Maturation (Protocol 2).

The values for mean contents of 17β-estradiol, progesterone and androgens in follicular fluid are presented in Figure 14. In control rats treated with 4 IU PMSG, the content of 17β-estradiol was 116.2±11.5 ng/ml at 24 hr and significantly (p<0.05) increased to the value of 265.7±62.8 ng/ml by 48 hr; no further increase was noted at 60 hr. In superovulated rats treated with 20 IU PMSG, the changes in 17β-estradiol content were comparable to control values throughout the period examined. Progesterone content in control rats rose steadily from 24 hr (260.6±21.2 ng/ml) to reach a maximum by 60 hr (6266.2±792.1 ng/ml). Although the changes in progesterone levels between control and superovulated rats followed similar patterns, there was a significant (p<0.05) difference in the levels at 48 hr: 2076.6±217.7 ng/ml in control and 4274.4±664.1 ng/ml in superovulated rats. A great difference between control and superovulated rats was characterized by different patterns of androgens observed at different times. Androgen content in control rats was 882.1±94.8 ng/ml at 24 hr and sharply declined to the level of 37.1±14.4 ng/ml by 60 hr. The content in superovulated rats was 186.4±33.0 ng/ml at 24 hr which was significantly (p<0.01) lower than control value. Thereafter, it significantly (p<0.05) rose to the value of 474.6±75.8 ng/ml by 48 hr and remained at this level. These values obtained at 48 hr or 60 hr were significantly (p<0.05) higher than controls.

As shown in Figure 15, in control rats, the mean ratios of androgens:17β-estradiol (A/E), progesterone:17β-estradiol (P/E) and progesterone:androgens (P/A) reflected sequential changes at various times.
Changes in follicular contents of 17β-estradiol, progesterone and androgens after administration of 4 or 20 IU PMSG to immature rats. Values are given as means ± SE (n=5-7). The means with no superscripts in common are significantly different (p<0.05).
The diagram shows the levels of three different hormones: 17β-estradiol, progesterone, and androgens, measured in follicular cells as a function of time after PMSG (Pituitary Menopause Syncytial Granulosa). The x-axis represents time after PMSG (hr), with points at 24, 48, and 60 hours. The y-axis represents follicular steroids (ng/ml).

For 17β-estradiol, the bars show the response to 4 IU PMSG (open bars) and 20 IU PMSG (shaded bars). At 24 hours, the response to 4 IU PMSG is labeled 'a', and for 20 IU PMSG, 'ab'. At 48 hours, the response is 'bc' for 20 IU PMSG and 'ab' for 4 IU PMSG. At 60 hours, the response is 'abc' for 20 IU PMSG and 'ab' for 4 IU PMSG.

For progesterone, the response to 4 IU PMSG is labeled 'a' at 24 hours, 'ab' at 48 hours, and 'cd' at 60 hours. The response to 20 IU PMSG is 'b' at 24 hours, 'c' at 48 hours, and 'cd' at 60 hours.

For androgens, the response to 4 IU PMSG is 'ab' at 24 hours, 'ab' at 48 hours, and 'bc' at 60 hours. The response to 20 IU PMSG is 'a' at 48 hours and 'bc' at 60 hours.

Statistical comparisons are indicated by different letters above the bars, with different letters indicating significant differences.
Changes in ratios of androgens/17β-estradiol, progesterone/17β-estradiol and progesterone/androgens in follicular fluid after administration of 4 or 20 IU PMSG to immature rats. Values are given as means ± SE (n=5-7). The means with no superscripts in common are significantly different (p<0.05).
Androgens/17β-estradiol

- 4 IU PMSG
- 20 IU PMSG

Progesterone/17β-estradiol

Progesterone/Androgens

Time after PMSG (hr)
In contrast, the overall ratios in superovulated rats were consistently steady over the time period of experiment. In control rats, the A/E ratio decreased sharply, but P/E and P/A ratios increased gradually from 24 hr onwards. Significant changes were observed at 48 hr for A/E ratio (p<0.01), and at 60 hr for P/E (p<0.05) and P/A ratios (p<0.01). The A/E and P/E ratios in superovulated rats were significantly (p<0.05) lower and higher than each ratio in control rats at 24 hr, respectively. Thereafter, these ratios in superovulated rats remained constant with no substantial changes. The P/A ratio in superovulated rats was consistently low over the whole period examined and comparable to the ratio observed at 24 or 48 hr in control rats. However, at 60 hr, it was significantly (p<0.01) lower than the ratio in control rats.

The various stages of nuclear maturation of oocytes recovered from oviducts at different times after PMSG are summarized in Table II. Morphologically degenerate oocytes in control rats (1/57 oocytes at 72 hr) and in superovulated (6/88 oocytes at 48 hr and 25/139 oocytes at 72 hr) were excluded from the meiotic evaluation. The proportion of oocytes actually analyzable for the classification of each stage was 73% in control rats and 67-91% in superovulated rats, since some oocytes lost or scattered their chromosomes by occasional rupture of cell membrane during the process of preparing the oocytes. In control rats, observation of oocytes revealed circular indentation or constriction of oocyte membrane and complete aggregation of chromosomes into polar body, typical of metaphase II (Fig. 16). However, in superovulated rats, it was actually not possible to distinguish between metaphase I and metaphase II because of inconsistent
### TABLE II

Effects of superovulatory treatment on nuclear maturation of oocytes recovered from oviducts in rats

<table>
<thead>
<tr>
<th>Time after PMSG (hr)</th>
<th># of oocytes recovered&lt;sup&gt;a&lt;/sup&gt;</th>
<th># of oocytes examined</th>
<th>Stages of Meiosis (%)</th>
<th>Prophase I</th>
<th>Anaphase I</th>
<th>Telophase I</th>
<th>Metaphase I/II</th>
<th>Undetermined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control&lt;sup&gt;b&lt;/sup&gt;</td>
<td>72</td>
<td>57(5/5)</td>
<td>56</td>
<td>-</td>
<td>1(1.8)</td>
<td>-</td>
<td>51(91.0)*</td>
<td>4(7.1)</td>
</tr>
<tr>
<td>Super-ovulation&lt;sup&gt;c&lt;/sup&gt;</td>
<td>24</td>
<td>15(2/5)</td>
<td>15</td>
<td>10(66.7)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5(33.3)</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>88(5/7)</td>
<td>82</td>
<td>19(23.2)</td>
<td>13(15.9)</td>
<td>-</td>
<td>39(47.6)</td>
<td>11(13.4)</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>139(6/6)</td>
<td>114</td>
<td>1(0.9)</td>
<td>51(44.7)</td>
<td>7(6.1)</td>
<td>45(39.5)</td>
<td>10(8.8)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number of rats exhibiting ovulations/total number of rats killed at each time are given in parentheses. Recovered oocytes with visible signs of degeneration were included in total counting.

<sup>b</sup>Rats were given 4 IU of pregnant mare serum gonadotropin (PMSG).

<sup>c</sup>Rats were given 20 IU PMSG.

*Oocytes showed consistently typical metaphase II configuration.
FIGURE 16

Typical configuration of nuclear maturation of oocytes recovered from oviducts after PMSG. (a) prophase I at 48 hr (20 IU PMSG). x400, (b) metaphase I at 72 hr (20 IU PMSG). x400, (c) telophase I at 72 hr (20 IU PMSG). x1000, (d) metaphase II at 72 hr (4 IU PMSG). x400
formation of typical polar body. Prior to or immediately after the completion of membrane abstriction, polar bodies in some portion of superovulated oocytes became rapidly degenerated. In control rats, of the 56 oocytes examined at 72 hr, only one oocyte was at anaphase I, but the majority (91.0%) showed metaphase II configuration. In contrast, superovulated oocytes displayed considerably different stages of meiotic maturation over the time period of experiment (Fig. 16). Although, of the 15 oocytes examined at 24 hr, 10 oocytes (66.7%) were consistently at prophase I except for the unanalyzable 5 oocytes, the oocytes superovulated at 48 or 72 hr were not synchronized to any stage of nuclear maturation. The percentages of oocytes at prophase I, anaphase I, telophase I and metaphase I/II were 23.2% (19/82 oocytes), 15.9% (13/82 oocytes), 0% and 47.6% (39/82 oocytes) at 48 hr, and 0.9% (1/114 oocytes), 44.7% (51/114 oocytes), 6.1% (7/114 oocytes) and 39.5% (45/114 oocytes) at 72 hr, respectively.
III. Serum LH Response (Protocol 3).

The time courses and patterns of LH release in the blood of control (4 IU PMSG) and superovulated (20 IU PMSG) rats are illustrated in Figure 17. Both groups showed a similar time relationship of biphasic LH response to PMSG. However, a great difference was noted in the magnitude of serum LH levels between the two groups.

Mean basal levels of serum LH prior to administration of PMSG were 0.37 to 0.50 ng/ml. Serum LH levels began to rise significantly (p<0.01) between 0 hr and 12 hr, and reached the first peaks of $1.81 \pm 0.21$ ng/ml at 12 hr and $3.78 \pm 0.18$ ng/ml at 18 hr in control and superovulated rats, respectively. Thereafter, the LH levels in both groups gradually tapered off by 54 hr. The first peaks were followed by enormous and significant (p<0.001) elevations of serum LH with the second peaks which were synchronized at 60 hr in both groups. The peak values of second elevations were $14.98 \pm 0.92$ ng/ml and $6.85 \pm 1.29$ ng/ml in control and superovulated rats, respectively. Subsequently, the LH levels in both groups rapidly fell by 66 hr with no further changes apparent at 72 hr.

As compared to controls, between 12 hr and 54 hr, serum LH levels in superovulated rats were significantly (p<0.001) elevated. This elevation was consistent at each time point. However, at 60 hr, the mean peak value of the second elevation in superovulated rats was significantly reduced by 54% below that of control rats. Similarly, a maximum increase of mean Δ LH between the two peaks was much less in superovulated rats than that in control rats. The mean Δ LH after subtraction of the first peak from the second peak was 3.07 ng/ml in superovulated rats and 13.17 ng/ml in control rats.
Changes in serum LH concentrations after administration of 4 or 20 IU PMSG to immature rats. Sequential blood samples were taken from the same animal using chronic catheterization into external jugular vein. Values at each point represent the means ± SE (n=7).
The first increases of serum LH were found to result from the cross-reaction of the injected PMSG with LH antibody in the assay procedure. The initial increases were dose-dependent with a prolonged half-life of 30-36 hr which was much longer than that for genuine LH (0.5 hr, reported by Bogdanove and Gay, 1969). In addition, the result of dilution binding of NIH-LH-RP2, PMSG and serum pools in the LH assay procedure (Fig. 18) showed that PMSG actually cross-reacted with LH antibody, and that the serial dilution curve of serum pools obtained from superovulated rats during the initial increase was not parallel to that of the NIH-LH-RP2 standard but was much closer to that of PMSG. On the other hand, the serial dilution curve of serum pools obtained from control rats at 60 hr was parallel to the NIH-LH-RP2 standard curve.

After final collection of blood samples at 72 hr, the ovulatory response and changes of ovarian weight in response to two different doses of PMSG were examined (Table III). In control rats, the total mean number of oocytes recovered was 7.6±0.5 oocytes per rat, and mean wet weight of paired ovaries was 32.8±1.9 mg per rat. On the other hand, a superovulatory dose of PMSG was shown significantly to increase the ovulatory response (36.0±8.5 oocytes per rat, p<0.05) and ovarian wet weight (68.7±8.2 mg per rat, p<0.01) above those obtained by a control dose of PMSG.
FIGURE 18

LH assay of PMSG and serum pools, showing the cross-reactivity of the injected PMSG with LH antibody.

. PMSG: Non-injected and diluted preparation.
. INTACT POOL: Serum pools collected from the diestrous adult rats without injection of PMSG.
. POOL C60: Serum pools collected from the immature rats at 60 hr after injection of a control dose (4 IU) of PMSG.
. POOL S12-24: Serum pools collected from the immature rats at 12, 18 and 24 hr after injection of a superovulatory dose (20 IU) of PMSG.
TABLE III

Oocyte recovery and ovarian weights 72 hr after administration of PMSG to immature rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of oocytes (mean ± SE)</th>
<th>Ovarian weight (mg) (mean±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 IU PMSG</td>
<td>7</td>
<td>7.6±0.5</td>
<td>32.8±1.9</td>
</tr>
<tr>
<td>20 IU PMSG</td>
<td>7</td>
<td>36.0±8.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>68.7±8.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>All individuals in each group exhibited ovulations.

<sup>b</sup><i>p</i><0.05 and <sup>c</sup><i>p</i><0.01, statistically significant compared to 4 IU PMSG-treated group.
IV. Effects of Flutamide on Oocyte Morphology and Embryo Development

(Protocol 4).

The ovulatory response in rats superovulated with 40 IU PMSG was unaffected by flutamide treatment (Table IV). Apart from one rat in the vehicle treatment group, which showed little evidence of ovarian stimulation at 48 hr, all rats in the vehicle or flutamide treatment groups ovulated consistently 48 to 72 hr after PMSG. The total number of oocytes recovered at each time varied considerably between animals in each treatment group (between 15 and 64 oocytes/rat). No significant differences were observed in the mean number of oocytes between vehicle- and flutamide-treated groups throughout the period examined, nor did the mean number of oocytes in each treatment group increase significantly after 48 hr.

No degenerate oocytes were found in any pre-treatment control rats which ovulated at 24 hr (8.5±0.5 oocytes/rat). Flutamide treatment in rats superovulated with PMSG significantly reduced the percentage of degenerate oocytes ovulated at 48 hr (p<0.01), 60 hr (p<0.01) and 72 hr (p<0.01), compared to the vehicle-treated rats (Table IV). The maximum decrease in degenerate oocytes was observed at 72 hr (26-fold); one or two degenerate oocytes were occasionally recovered from three of the six flutamide-treated rats.

The changes in ovarian contents of androgens, 17β-estradiol and progesterone after treatment with vehicle or flutamide are shown in Figure 19. By 48 hr, all the three ovarian steroid hormones in both groups of treated rats were significantly (p<0.01) elevated above the values of pre-treatment controls obtained at 24 hr. Compared to the vehicle regimen,
<table>
<thead>
<tr>
<th>Time after PMSG (hr)$^a$</th>
<th>48</th>
<th>60</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vehicle$^b$</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of oocytes</td>
<td>31.0±7.0</td>
<td>31.1±1.9</td>
<td>34.5±2.9</td>
</tr>
<tr>
<td>% degenerate oocytes</td>
<td>19.0±5.0</td>
<td>54.7±3.5</td>
<td>41.7±4.2</td>
</tr>
<tr>
<td><strong>Flutamide$^c$</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of oocytes</td>
<td>35.4±5.6</td>
<td>27.1±2.1</td>
<td>39.2±4.9</td>
</tr>
<tr>
<td>% degenerate oocytes</td>
<td>2.1±0.8$^d$</td>
<td>19.3±1.4$^e$</td>
<td>1.6±0.7$^f$</td>
</tr>
</tbody>
</table>

$^a$ Results are expressed as means ± SEM for the number of ovulated rats ($n = 6$ or $7$).

$^b,c$ Rats were treated with vehicle or $5$ mg flutamide at $30$ and $36$ hr after injection of $40$ IU PMSG.

$^d-f$ $p<0.01$, compared to corresponding vehicle treatment group.
FIGURE 19

Ovarian contents of androgens, 17β-estradiol, and progesterone after treatment with vehicle or flutamide in 40 IU PMSG-injected rats. Values are means ± SEM for paired ovaries (n=6 or 7). **p<0.01, compared to pre-treatment controls (40 IU PMSG only). α,βp<0.01 and γp<0.05, compared to corresponding vehicle treatment group.
Androgens

- 401U PMSG only
- 401U PMSG + Veh
- 401U PMSG + Flu

17β-estradiol

Progesterone

Time after PMSG (hr)
flutamide treatment was associated with a 58% decrease in androgen level (p<0.01), a 64% decrease in 17β-estradiol level (p<0.01) and a 30% increase in progesterone level (p<0.05) at 60 hr. There were no significant differences in all three ovarian steroid hormones between vehicle- and flutamide-treated groups before and after 60 hr. With the exception of 17β-estradiol, changes in serum steroid hormones followed comparable profiles to those determined for ovarian tissues throughout the intervals examined (Figs. 19 and 20).

Data for the recovery and preimplantation development of superovulated ova are presented in Table V. The number of ova recovered from superovulated rats treated with vehicle diminished rapidly after Day 2 and reached 0 on Day 5 of pregnancy. In contrast, 8 to 12 embryos were consistently recovered from control animals over the same time period. Flutamide treatment did not prevent the early loss of preimplantation embryos which took place after Day 2, although on Day 5 embryos were occasionally recovered from the flutamide-treated rats. There were no significant differences between the two groups treated with vehicle or flutamide in the absolute number of ova recovered from Days 2 to 5. Compared to control rats, embryo development in both vehicle- and flutamide-treated rats was retarded consistently from Day 2, and maximum cleavage was arrested from Day 3 onwards. Embryos in the flutamide treatment group developed to a maximum of 6-cells, while those in the vehicle treatment group did not develop beyond the 4-cell stage.

The ova with visible signs of degeneration were consistently recovered from both oviducts and uteri of the vehicle- or flutamide-treated
Serum levels of androgens, 17β-estradiol, and progesterone after treatment with vehicle or flutamide in 40 IU PMSG-injected rats. Values are means ± SEM (n=6 or 7). **p<0.01, compared to pre-treatment controls (40 IU PMSG only). αp<0.01 and βγp<0.05, compared to corresponding vehicle treatment group.
TABLE V

Effects of flutamide on recovery and developmental capacity of superovulated ova in preimplantation stage

<table>
<thead>
<tr>
<th>Day of pregnancy</th>
<th>2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>3&lt;sup&gt;b&lt;/sup&gt;</th>
<th>4&lt;sup&gt;c&lt;/sup&gt;</th>
<th>5&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4 IU PMSG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of ova recovered</td>
<td>9.8±0.5</td>
<td>10.2±0.5</td>
<td>10.3±0.3</td>
<td>0</td>
</tr>
<tr>
<td>oviduct</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9.5±0.7</td>
</tr>
<tr>
<td>uterus</td>
<td>2-cell</td>
<td>2-to 6-cell</td>
<td>8-cell</td>
<td>blastocyst</td>
</tr>
<tr>
<td>Developmental stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>40 IU PMSG plus vehicle</strong></td>
<td>22.7±3.8</td>
<td>13.2±3.1</td>
<td>1.2±0.5</td>
<td>0</td>
</tr>
<tr>
<td>No. of ova recovered</td>
<td>0.8±0.7</td>
<td>1.7±0.7</td>
<td>0.8±0.7</td>
<td>0</td>
</tr>
<tr>
<td>oviduct</td>
<td>1-to 2-cell</td>
<td>1-to 4-cell</td>
<td>1-to 4-cell</td>
<td></td>
</tr>
<tr>
<td>uterus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Developmental stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>40 IU PMSG plus flutamide</strong></td>
<td>21.0±3.1</td>
<td>16.3±2.5</td>
<td>2.3±1.0</td>
<td>1.4±1.2</td>
</tr>
<tr>
<td>No. of ova recovered</td>
<td>0.5±0.5</td>
<td>3.7±1.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>oviduct</td>
<td>1-to 2-cell</td>
<td>1-to 6-cell</td>
<td>2-to 6-cell</td>
<td>4-to 6-cell</td>
</tr>
<tr>
<td>uterus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Developmental stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a-d</sup> Results are expressed as means ± SEM for the number of pregnant rats: (a-c) 6 rats, and (d) 5 rats each group.

<sup>e,f</sup> Rats were treated with vehicle or 5 mg flutamide at 30 and 36 hr after injection of 40 IU PMSG.

<sup>g-j</sup> No significant difference (p>0.05), compared to corresponding vehicle treatment group.
rats from Day 2 to 5 (Table VI). In contrast, no degenerate ova in control rats (4 IU PMSG) were found throughout the examination period. In superovulated animals, compared to the vehicle group, flutamide treatment significantly (p<0.01) decreased the percentage of degenerate ova on Day 2. However, after Day 3, this percentage in the flutamide-treated rats rapidly increased to reach a maximum of 83.4% on Day 5 which was similar to that in the vehicle-treated rats on Day 4. On the other hand, flutamide treatment significantly (p<0.01) increased the percentage of cleaved embryos above that from the vehicle-treated rats on Day 2 (Table VI). However, after Day 3, the percentage of cleaved embryos in the flutamide-treated rats gradually decreased to a low of 16.7% on Day 5, which was comparable to that in the vehicle-treated rats on Day 4.

Serum androgens and progesterone from Day 2 to 5 (p<0.01) and serum 17β-estradiol on Day 2 (p<0.05) in superovulated rats treated with vehicle were significantly elevated above the values in the control rats treated with 4 IU PMSG (Fig. 21). Compared to the results of vehicle regimen, flutamide treatment after 40 IU PMSG decreased serum androgen level by 39% on Day 2 (p<0.05). On Day 2, serum 17β-estradiol in the flutamide-treated rats greatly decreased by 51% below that in the vehicle-treated rats but the difference was not statistically significant.

Data obtained from a separate experiment show the changes in serum contents of androstenedione, testosterone and 5α-dihydrotestosterone after treatment of a control dose (4 IU) of PMSG alone and a superovulatory dose (20 IU) of PMSG with vehicle or flutamide (Fig. 22). Throughout the examination period, the values of all three androgenic steroids in the
Effects of flutamide on normality and cleavage of superovulated ova from both oviducts and uterine horns in preimplantation stage.

<table>
<thead>
<tr>
<th>Day of pregnancy</th>
<th>2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>3&lt;sup&gt;b&lt;/sup&gt;</th>
<th>4&lt;sup&gt;c&lt;/sup&gt;</th>
<th>5&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vehicle</strong>&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% degenerate ova</td>
<td>45.9±6.0</td>
<td>35.7±12.2</td>
<td>76.3±17.7</td>
<td>-</td>
</tr>
<tr>
<td>% cleaved embryos</td>
<td>13.2±4.4</td>
<td>37.0±10.1</td>
<td>17.5±11.8</td>
<td>-</td>
</tr>
<tr>
<td><strong>Flutamide</strong>&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% degenerate ova</td>
<td>12.6±5.0</td>
<td>13.4±5.6</td>
<td>41.7±25.0</td>
<td>83.4±16.7</td>
</tr>
<tr>
<td>% cleaved embryos</td>
<td>73.6±6.7&lt;sup&gt;h&lt;/sup&gt;</td>
<td>75.6±6.5</td>
<td>58.3±25.0</td>
<td>16.7±16.7</td>
</tr>
</tbody>
</table>

<sup>a-d</sup> Results are expressed as means ± SEM for the number of pregnant rats in which more than one ovum were recovered: (a,b) 6 rats, (c) 4 rats, and (d) 2 rats each group.

<sup>e,f</sup> Rats were treated with vehicle or 5 mg flutamide at 30 and 36 hr after injection of 40 IU PMSG.

<sup>g,h</sup> p<0.01 within each parameter, compared to corresponding vehicle treatment group.
FIGURE 21

Serum levels of androgens, 17β-estradiol, and progesterone on Days 2 to 5 of pregnancy after administration of 4 IU PMSG alone and 40 IU PMSG plus vehicle or flutamide to immature rats. Values are means ± SEM (n=5 or 6). *p<0.05 and **p<0.01, compared to corresponding controls (4 IU PMSG). αp<0.05, compared to corresponding vehicle treatment group.
Androgens

- 41U PMSG
- 40IU PMSG*Veh
- 40IU PMSG+F

Day of Pregnancy

17β-estradiol

Progesterone

Day of Pregnancy

Serum Steroids (ng/mL)
FIGURE 22

Contents of serum androgenic steroids, androstenedione, testosterone, and 5α-dihydrotestosterone after administration of 4 IU PMSG alone and 20 IU PMSG plus vehicle or flutamide to immature rats. Values are means ± SEM (n=10). *p<0.05 and **p<0.01, compared to pre-treatment controls (20 IU PMSG only). α,β,γp<0.05, compared to corresponding vehicle treatment group at each time.
Androstenedione

- 4IU PMSG
- 20IU PMSG only
- 20IU PMSG+Veh
- 20IU PMSG+Flu

Time after PMSG (hr)

Testosterone

5α-dihydrotestosterone

Serum Androgenic Steroids (ng/ml)

Time after PMSG (hr)
control rats (4 IU PMSG) decreased steadily from the maximum values at 24 hr (androstenedione, 1.53±0.17 ng/ml; testosterone, 1.67±0.31 ng/ml; 5α-dihydrotestosterone, 0.56±0.08 ng/ml) to their respective minimum values by 60 hr (androstenedione, 0.04±0.03 ng/ml; testosterone 0.08±0.04 ng/ml; 5α-dihydrotestosterone, 0.06±0.02 ng/ml). In contrast, the profile of androgenic steroids in the vehicle-treated superovulated rats exhibited generally increasing trend from 24 hr. Compared to the steroid levels in pre-treatment control rats of superovulatory regimen at 24 hr, testosterone and 5α-dihydrotestosterone contents significantly (p<0.01) increased by 48 hr, while androstenedione content showed a significant (p<0.05) elevation by 60 hr. Flutamide treatment to the superovulated rats significantly (p<0.05) decreased the contents of aromatizable androgenic steroids below those of vehicle regimen: androstenedione by 76% at 48 hr; testosterone by 75% at 48 hr and by 87% at 60 hr. The lower levels of nonaromatizable androgen, 5α-dihydrotestosterone were obtained from the rats of vehicle treatment group, as compared to those of flutamide regimen, but not statistically significant.
V. Effects of RU486 and Tamoxifen on Ovulation and Oocyte Morphology (Protocol 5).

Effects of the steroid antagonists (RU486 and tamoxifen) on ovulation, oocyte normality and ovarian weight in rats injected with a control dose (4 IU) of PMSG are presented in Table VII. All rats in vehicle-treated group ovulated a range of 9-14 oocytes per rat. The ovulatory response to 4 IU PMSG was apparently inhibited by the treatment with either RU486 or tamoxifen. In RU486-treated group, although the proportion of rats ovulating seemed to be slightly reduced by 14.3%, the mean number of oocytes recovered from each rat was significantly (p<0.01) reduced by 39.3% as compared to vehicle-treated group. In tamoxifen-treated group, both the proportion of rats ovulating and the mean number of oocytes per rat were significantly reduced by 71.4% and 94.9% (p<0.001), respectively, as compared to each parameter observed from vehicle-treated group of animals. Thus, tamoxifen seemed to be more effective in the inhibition of ovulatory response than RU486 when administered to 4 IU PMSG-injected rats at equipotent doses (three consecutive injections of 1 mg per animal).

More than 95% of oocytes recovered from the rats of vehicle-treated group appeared to be normal; only one or two degenerate oocytes were occasionally recovered from three of the seven vehicle-treated rats. No significant difference was observed in gross morphology of the oocytes recovered from the two groups of rats treated with vehicle or RU486 after 4 IU PMSG. In contrast, tamoxifen treatment significantly (p<0.001) increased the proportion of oocytes exhibiting visible signs of degeneration as compared to that in vehicle-treated group of animals.
### TABLE VII

Effects of RU486 and tamoxifen on recovery and normality of oocytes from oviducts, and ovarian weight in 4 IU PMSG-injected rats\(^a\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Proportion of rats ovulating</th>
<th>No. of oocytes(^b)</th>
<th>% degenerate oocytes(^c)</th>
<th>mg ovarian tissue(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>7/7</td>
<td>11.7±0.7</td>
<td>4.7±2.3</td>
<td>43.7±3.9</td>
</tr>
<tr>
<td>RU486</td>
<td>6/7</td>
<td>7.1±1.3(^e)</td>
<td>7.5±4.8</td>
<td>50.3±4.8</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>2/7</td>
<td>0.6±0.4(^f)</td>
<td>75.0±25.0(^g)</td>
<td>29.3±2.0(^h)</td>
</tr>
</tbody>
</table>

\(^a\)Rats were treated with vehicle, 1 mg RU486 or 1 mg tamoxifen at 24, 36 and 48 hr after injection of 4 IU PMSG.

\(^b\)-\(^d\)Results obtained 24 hr after treatment are expressed as means ± SE.

\(^e_p<0.01, \(^f_p<0.001, \(^g_p<0.05, \)compared to corresponding vehicle-treated group.
Ovarian weight response to 4 IU PMSG was not affected by RU486 treatment, since there were no significant differences in the mean weight of ovarian tissues between vehicle- and RU486-treated groups of rats. In contrast, tamoxifen treatment significantly (p<0.05) reduced the mean ovarian weight by 33.0% as compared to that in vehicle-treated group.

The levels of serum 17β-estradiol, progesterone and androgens after the treatment with vehicle, RU486 or tamoxifen in 4 IU PMSG-injected rats are illustrated in Figure 23. There were no significant differences in the levels of all three serum steroid hormones between vehicle- and RU486-treated groups of rats. However, compared to vehicle-treated group, tamoxifen treatment was associated with a 200.0% increase in 17β-estradiol level (p<0.05) and a 40.3% decrease in progesterone level (p<0.05).

Effects of the RU486, and tamoxifen on ovulation, oocyte normality, and ovarian weight in rats injected with a superovulatory dose (40 IU) of PMSG are shown in Table VIII. Although no ovulations were observed in any rats of pre-treatment control group, all rats in the vehicle-treated group ovulated consistently through 60 to 72 hr after 40 IU PMSG. The mean number of oocytes recovered from the rats of the vehicle-treated group was 24.4±1.4 oocytes per rat at 60 hr and further increased to reach 40.1±2.5 oocytes per rat at 72 hr. It was evident that the superovulatory response to 40 IU PMSG was inhibited by the treatment with RU486. In RU486-treated group, the proportion of rats exhibiting ovulation was reduced by 42.9% at 60 hr and 11.1% at 72 hr as compared to vehicle-treated group at each time. The mean number of oocytes recovered from each rat of this group was significantly (p<0.01) reduced by 70.1% at 60 hr and 47.1% at 72 hr when compared to the
FIGURE 23

Serum levels of 17β-estradiol, progesterone and androgens 24 hr after treatment with vehicle, RU486 or tamoxifen in 4 IU PMSG-injected rats. Values are means ± SE (n=7). The means with no superscripts in common are significantly different (p<0.05).
TABLE VIII

Effects of RU486 and tamoxifen on recovery and normality of oocytes from oviducts, and ovarian weight in 40 IU PMSG-injected rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time after PMSG (hr)</th>
<th>60</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. oocytes</td>
<td>% degenerate oocytes</td>
<td>mg ovarian tissue</td>
</tr>
<tr>
<td>Vehicle</td>
<td>24.4±1.4 (7/7)C</td>
<td>50.4±9.8</td>
<td>147.7±8.2</td>
</tr>
<tr>
<td>RU486</td>
<td>7.3±2.8d (4/7)</td>
<td>45.1±6.7</td>
<td>142.9±6.0</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>22.6±1.2 (5/5)</td>
<td>73.2±10.8</td>
<td>121.1±4.7f</td>
</tr>
</tbody>
</table>

*Rats were treated with vehicle, 1 mg RU486 or 2.5 mg tamoxifen at 24, 36 and 48 hr after injection of 40 IU PMSG.*

*Results are expressed as means± SE.*

*Number of rats exhibiting ovulations/total number of rats killed at each time are given in parentheses.*

*d,e,g&lt;0.01, f&lt;0.05, h,i&lt;0.001, compared to corresponding vehicle-treated group.*
vehicle-treated group at each time. On the other hand, tamoxifen was marginally effective for inhibition of superovulatory response, although it was administered to the 40 IU PMSG-injected rats at even higher doses (three consecutive injections of 2.5 mg per animal) than RU486. When compared to the vehicle-treated group, a significant difference was noted in the mean number of oocytes only at 72 hr. In this group, all rats ovulated consistently throughout the period examined.

Relatively high proportions of degenerate oocytes were observed in vehicle-treated superovulated rats: 50.4±9.8% at 60 hr and 41.6±5.1% at 72 hr after 40 IU PMSG. There were no significant differences in the percentages of degenerate oocytes recovered from the rats in vehicle- and RU486-treated groups throughout the period examined. In contrast, tamoxifen treatment significantly (p<0.001) increased the proportion of oocytes exhibiting signs of degeneration at 72 hr as compared to that in vehicle-treated group of animals.

The weight response of superovulated ovaries after 40 IU PMSG was not affected by RU 486 treatment but markedly inhibited by tamoxifen treatment. Virtually no significant differences were recorded in the mean ovarian weight between vehicle- and RU486-treated group of rats. In contrast, tamoxifen treatment significantly reduced the mean ovarian weight by 18.0% at 60 hr (p<0.05) and 26.7% at 72 hr (p<0.001) as compared to that in vehicle-treated group of animals.

The changes in serum levels of 17β-estradiol, progesterone and androgens before or after the treatment with vehicle, RU486 or tamoxifen in rats injected with 40 IU PMSG are presented in Figure 24. At 60 hr after
FIGURE 24

Serum levels of 17β-estradiol, progesterone and androgens before or after treatment with vehicle, RU486 or tamoxifen in 40 IU PMSG-injected rats. Values are means ± SE (n=5-7). The means with no superscripts in common are significantly different (p<0.05).
17β-estradiol

- 40IU PMSG only
- 40IU PMSG+Veh
- 40IU PMSG+RU486
- 40IU PMSG+Tam

Progesterone

Serum Steroids (ng/ml)

Androgens

Time after PMSG (hr)
PMSG, the levels of all three steroid hormones in vehicle-treated group of superovulated rats were significantly (p<0.05) elevated above those of pre-treatment controls obtained at 12 hr. Compared to the vehicle-treated group, there were no significant changes in any of the three steroid hormones throughout the period examined after RU486 treatment. However, tamoxifen treatment resulted in marked alterations in the levels of overall steroid hormones: 102.7% (p<0.01) and 800.0% (p<0.001) increases for 17β-estradiol, 63.9% (p<0.05) and 180.6% (p<0.001) increases for androgens at 60 hr and 72 hr, respectively, and a 46.2% (p<0.05) decrease for progesterone at 60 hr.
DISCUSSION

Despite widespread application of superovulation techniques for the yield of multiple oocytes and embryos in livestock industry and human IVF and embryo replacement programmes, superovulatory treatment with exogenous gonadotropins frequently results in impaired fertility such as reduced fertilization, abnormal embryo development and implantation failure in various mammalian species. This impairment has previously been associated with variability in time of ovulation (Evans and Armstrong, 1983; Walton et al., 1983) and excessive estrogenic stimulation of reproductive tract to result in asynchrony between embryonic and uterine environment (Miller and Armstrong, 1981b; Walton and Armstrong, 1982). However, major factors and critical time involved in the reduced fertility following superovulation were not fully understood. The defects could occur in the process of the oocyte maturation through ovulation prior to fertilization by hyperstimulation of ovarian tissues and acceleration of follicular development because of the biochemical properties of PMSG with its prolonged action and a mixture of FSH and LH to stimulate follicular steroid secretion (Gospodarowicz and Papkoff, 1967; Schams et al., 1978). Pre- or peri-ovulatory endocrine aspects including the secretion of follicular steroids and endogenous gonadotropins are believed to affect the final maturation and further fertile life of oocytes prior to fertilization. In the present study conducted with five experimental protocols, the primary defects and possible mechanisms leading to infertility following superovulation have been discussed with special attention to the ovulatory response, oocyte quality (gross morphology and nuclear maturation),
developmental potential of preimplantation embryos, and pre- or peri-ovulatory responses of ovarian steroid hormones and circulating LH in the rats superovulated with PMSG.

Protocol 1. The results of this experiment show that a single injection of superovulatory doses (20 and 40 IU) of PMSG in rats induced the first ovulation as early as 24 hr with two distinct increases in the number of released oocytes: one before 36 hr and the other after 48 hr of treatment. In addition, superovulatory treatments caused a dose-dependent increase in the population of abnormal oocytes and a marked alteration of ovarian steroid pattern from 36 hr onwards after gonadotropin administration.

Time-course studies of ovulation using various neuropharmacologic central nervous system depressants (Sorrentino et al., 1972; Hillensjo et al., 1974) and hypophysectomy (Strauss and Meyer, 1962; Zarrow and Quinn, 1963) suggested that in immature rats the release of endogenous LH occurs at 53-57 hr following PMSG injection. The present results, however, revealed that a single injection of 20 or 40 IU PMSG caused the first ovulation 24 hr after treatment. Walton et al. (1983) mentioned the occurrence of the first ovulation 38-46 hr after injection of 40 IU PMSG in immature rats. The variation of the first ovulation induction time may be due to the different model system, i.e. it may depend upon body weight or age of rats. The occurrence of the first ovulation as early as 24 hr is evidenced by the observation of ruptured and luteinized follicles in the stained sections of ovaries from rats sacrificed 24 hr after injection of 20 or 40 IU PMSG. These observations indicate that 20 or 40 IU PMSG may have contained sufficient LH-like activity to trigger ovulation as early as 24 hr after injection.
The precise mechanism of PMSG-induced ovulation is not known. According to Schams et al. (1978), PMSG has a long half-life and a slow clearance rate as a consequence of its very high sialic acid content, as well as a mixture of FSH- and LH-like activity when measured by bioassay. These chemical properties of PMSG may be responsible for a premature activation of oocytes, especially in those follicles that show the greatest degree of receptor differentiation at the time of gonadotropin administration (Moor et al., 1985). It has been suggested that PMSG induces ovulation through both direct and indirect mechanisms in immature rats (McCormack and Meyer, 1963). Because of its dominant and prolonged FSH activity, PMSG primarily acts at ovarian follicular level to stimulate follicular development presumably by increasing the recruitment from non-growing pool of primordial follicles, and enhances ovarian steroid hormone production (Chatterjee et al., 1977; Dott et al., 1979). Subsequently, the increased steroids produce either a positive or a negative feedback on the endogenous LH surge to trigger ovulation (Ying and Meyer, 1969). In the present experiment, superovulatory treatment with 20 or 40 IU PMSG showed two different patterns: increases of ovulation between 24-36 hr and between 48-72 hr but a plateau between 36-48 hr after injection. The number of oocytes recovered during the first increasing period was less than or similar to normal recovery (up to 15 oocytes/rat), but above that in the second increasing period (up to 58 oocytes/rat). This finding indicates that the responses to superovulatory doses of PMSG take place possibly by different mechanisms depending upon the time after the treatment with PMSG. Ovarian steroid hormones are, in general, crucial in regulating ovulatory
response. The circulating level of estrogens produced by PMSG exerts its stimulatory effect on the hypothalamus and further, progesterone may synergize with estrogens to facilitate gonadotropin release in immature female rats (Hagino and Goldzieher, 1970). This facilitating action of progesterone may be due to its lowering the threshold of the hypothalamus for gonadotropin release (Kawakami and Sawyer, 1959). In the present experiment, the first increase of ovulation within 36 hr following superovulation appears to be due to the LH-like moiety of PMSG itself. This concept may be accounted for by the absence of significant elevation of 17β-estradiol and progesterone levels during the first stage. However, the second increase from 48 hr onwards may be due to the endogenous LH surge via positive feedback stimulus initiated by coincidental elevation of the steroids or due to both endogenous and exogenous LH activities. This gonadotropin has a biological half-life of 54-60 hr (Sasamoto et al., 1972). Therefore, after the first stage of increase, large superovulatory doses of PMSG may stimulate the growth of ovarian follicles to ovulate by its own LH-like moiety.

It has been suggested that high dose of PMSG increases ovarian steroidogenic efficiency, and produces premature activation of oocytes most probably by its sufficient LH-like activity (Moor et al., 1985). This earlier activation of oocytes may cause abnormalities because of the likely unsuitable follicular environment for their maturation and over-ripeness at ovulation. In superovulated immature rats, excessive rises in both ovarian and serum steroid levels (mainly 17β-estradiol) were noted prior and subsequent to normally expected time of ovulation (Miller and Armstrong,
Walton and Armstrong (1981) suggested that the superovulatory treatment with 40 IU PMSG, because of its prolonged biological activity, strikingly elevated estrogen levels for longer than normal periods. However, no published data are available on the ovarian androgen response during the extended period of ovulation. Further, the correlation between gonadotropin-induced changes of overall ovarian steroid responses (estrogens, progesterone and androgens) and oocyte abnormalities following superovulation has not yet been studied.

The results of the present experiment suggest that the alterations in ovarian steroid levels following superovulation may be responsible for the occurrence of oocyte degeneration 36-48 hr after administration of 20 or 40 IU PMSG. From this period onwards, superovulatory treatments with PMSG produce an early rise or prolonged elevation of 17β-estradiol and progesterone above control levels. In addition, two peaks of androgens in control rats occur before this period, whereas in superovulated rats, there was a persistent elevation with similar magnitude to control regimen after this period. The alterations of overall steroid patterns from 36 hr onwards coincide with the timing of rising percentage of abnormal oocytes. The abnormality of oocytes is consistent with the observation of a predominance of oocyte degeneration in the large follicular sections from superovulated rats sacrificed 48 hr after PMSG injection. A portion of degenerating oocytes previously trapped in large follicles may contribute to ovulating pool of the abnormal oocytes. This possibility is supported by the suggestion of Fleming (1982) that superovulatory treatment may induce the ovulation of degenerating oocytes from the abnormal follicles, which would
not ovulate in normal cyclic estrous stage. Additionally, oocyte aging due to earlier activation by the LH activity of hPMSG may also be a contributing factor for the occurrence of abnormal oocytes. This may be accounted for by the present results demonstrating that 36 hr after administration of 40 IU hPMSG, a portion of abnormal oocytes free of cumulus masses were observed despite no significant elevation of 17β-estradiol and androgen levels above control. In the present experiment, no assessment was made of fertilizability and developmental capacity of "normal-appearing" superovulated oocytes, but such oocytes would still likely have these potentials. Walton and Armstrong (1983) demonstrated that freshly ovulated "normal-appearing" oocytes following superovulation with 40 IU hPMSG have an equal ability to fertilize and develop to fetal stage as do oocytes from 4 IU hPMSG-treated rats.

The participation of steroids in the maturation of mammalian oocytes in vitro has previously been documented. 17β-estradiol, at a variety of concentrations, significantly reduced the incidence of oocyte maturation in rabbit and cattle (Robertson and Baker, 1969), pig (McGaughey, 1977), and mouse (Nekola and Smith, 1974; Eppig and Koide, 1978). Progesterone, at a high concentration (102 μM), enhanced the oocyte maturation in rabbit and cattle (Robertson and Baker, 1969), and gonadotropin-treated prepubertal rhesus monkey (Cook and Graham, 1976), while at low concentrations (10 and 31.8 μM) it decreased maturation frequency in mouse (Nekola and Smith, 1974; Eppig and Koide, 1978) and rabbit (Smith et al., 1978). Moor et al., (1980b) demonstrated that alterations in the normal pattern of steroids secreted during maturation
induced intracellular changes in sheep oocytes expressed as gross abnormalities at fertilization. In Nembutal-treated rats, prolonged exposure of the intrafollicular oocytes to 17β-estradiol during the ovulatory delay is causally related to subsequent abnormal development and embryonic death (Butcher and Pope, 1979). The mechanism by which estrogen induces abnormal embryos is unknown but related to alterations in the oocyte prior to fertilization (Peluso and Butcher, 1974). The results of present experiment have revealed the rising percentage of abnormal oocytes following superovulation is dose-dependent. However, 17β-estradiol level in 20 IU PMSG-treated rats was consistently higher than that in 40 IU PMSG-treated rats. Explanation of this phenomenon needs further experimentation. This raises the question of whether the prolonged elevation of 17β-estradiol in superovulated rats might be a major factor leading to the incidence of abnormal oocytes from 36 hr after injection of PMSG. In the present experiment, the most interesting result is the significant positive correlation between ovarian or serum androgen levels and percentages of abnormal oocytes between 24 and 60 hr following superovulation.

Protocol 2. The steroid patterns in follicular fluid of control (4 IU PMSG) and superovulated rats (20 IU PMSG) closely resemble the changes in ovarian tissues and serum reported previously (Yun et al., 1987) indicating that superovulatory treatment results in abnormal follicular steroidogenesis. In the present experiment, superovulation with 20 IU PMSG in immature rats substantially altered the follicular contents of progesterone and particularly androgens, and consistently interfered with sequential changes in overall ratios of A/E, P/E and P/A. Additionally, it
produced atypical ovulations of meiotically premature or asynchronous oocytes.

In immature rats, the doses of 4-8 IU PMSG are known to produce a pattern of circulating steroid hormones similar to that seen in cycling adult rats, and induce an LH surge which generates a physiological number of ovulating follicles (Wilson et al., 1974; Parker et al., 1976). Time course studies of ovulatory response in rats demonstrated the critical time of LH surge at 53-57 hr (Sorrentino et al., 1972; Hillensjo et al., 1974) and ovulation at 60-72 hr after PMSG (Walton et al., 1983; Yun et al., 1987). Follicular oocytes obtained from PMSG-injected rats showed a resumption of meiosis, based on germinal vesicle break-down, 2-3 hr after LH surge (Hillensjo et al., 1974). Therefore, it is most likely that in immature rats low doses (4-8 IU) of PMSG induce ovulations with integrated sequential changes in endocrine response as well as with synchronized process of oocyte maturation. This concept could be supported by the current observations of consistent recovery of metaphase II oocytes at ovulation and sequential changes in follicular steroid contents in control rats treated with 4 IU PMSG. Follicular steroid profile, except for androgens, in control rats generally accords with the results of serum steroids (Parker et al., 1976).

In the present experiment, the follicular contents of both 17β-estradiol and progesterone from control rats were significantly elevated by 48 hr after PMSG. The elevations of these follicular steroids prior to the expected critical time are believed to trigger or stimulate the preovulatory gonadotropin surge as suggested by Hagino and Goldzieher (1970). On the other hand, the current observation of change in follicular androgen content
does not confirm the profile of consistent elevation of testosterone as reported by Parker et al (1976). A great difference in androgen response to PMSG may be due to the difference in androgenic steroids measured; in the present study, the steroids measured using testosterone antiserum were referred to as androgens because of its high cross-reactivity with other androgens. The role of initial stimulation and subsequent suppression of androgen secretion during follicular development in control rats remains to be clarified.

The results of present experiment suggest that abnormal follicular steroidogenesis following superovulation may be associated with atypical ovulations of oocytes with meiotic aberration. Superovulatory treatment with 20 IU PMSG in rats resulted in significant elevation of progesterone, and initial decrease and subsequent increase in androgens as compared to control, and consistently disrupted the sequential changes with progressive decrease in A/E ratio and progressive increases in P/E and P/A ratios observed in control rats. Like the abnormal steroidogenesis, superovulated oocytes were subject to a high degree of prematurity or asynchronous nuclear maturation varying from prophase I to metaphase II. These results indicate that oocyte and follicle maturation in superovulated rats may occur independently of the normal physiological events. It has previously been demonstrated that superovulatory doses (20 and 40 IU) of PMSG in rats produced multiple ovulations which accompany the first ovulation as early as 24 hr after treatments (Yun et al., 1987). Such atypical ovulation has also been observed in gonadotropin-treated mature mice (Stern and Scheutz, 1970), and in progesterone- and PMSG-treated rats (Zarrow and Gallo, 1969; Gallo and Zarrow, 1970).
The asynchrony phenomenon has previously been reported in superovulated sheep (Moor et al., 1984) and cattle (Greve et al., 1984), and ascribed to premature meiotic activation of oocytes from certain follicles and meiotic arrest after germinal vesicle break-down and prior to or at the diakinesis stage (Moor et al., 1973). However, in these studies, the observation of asynchronous nuclear maturation following superovulation has been made in intrafollicular oocytes prior to ovulation. In the present experiment, the oviductal recovery of superovulated oocytes with various stages of nuclear maturation provides direct evidence of atypical ovulations after PMSG, and supports the previous suggestion that a portion of superovulated oocytes can be derived from physiologically unfit follicles, which would not ovulate at a regular cyclic estrus (Braw and Tsafriri, 1980; Fleming, 1982; Yun et al., 1987). The precise mechanism for the atypical ovulations of superovulated oocytes with meiotic aberration is not yet clear. By far, the precocious or multiple ovulations resulting from superovulatory treatments have been attributed to the exogenous LH activity of high doses of PMSG (De La Lastra et al., 1972; Miller and Armstrong, 1981b; Yun et al., 1987).

Developmental potential of superovulated oocytes with various stages of nuclear maturation was not assessed in the present experiment. However, a portion of these oocytes is likely to be susceptible to hostile oviductal environment presumably influenced by altered follicular steroid response, and may contribute to the pool of progressive increase in degenerate oocytes observed in the current and previous experiments (Yun et al., 1987) or to the reduced fertilization rate (Walton et al., 1983).
In conclusion, the results of present experiment provide direct evidence of atypical ovulations of superovulated oocytes with premature or asynchronous nuclear maturation which may contribute to the pool of abnormal oocytes prior to fertilization in rats after PMSG. Current data also indicate a close association between the incidence of meiotically aberrant oocytes and abnormal follicular steroidogenesis following superovulation with PMSG in rats.

Protocol 3. In the present experiment, both a control dose (4 IU) and a superovulatory dose (20 IU) of PMSG elicited two distinct LH peaks in rats: a first slight rise at 0-18 hr and a second sharp rise at 54-60 hr after treatment. This result, in general, agrees with the patterns produced by a different dose (10 IU) of PMSG (Hillensjo et al., 1974). Together, these findings indicate a similar and dose-independent time relationship of PMSG to induce LH surge. However, by analyzing the magnitude of the LH response, a superovulatory dose of PMSG was associated with a significant attenuation of the endogenous LH surge accompanied by prolonged elevations of serum LH, as compared to that corresponding to control regimen.

The present observation of two LH increases coupled with previous findings of two distinct ovulations after superovulatory treatment in rats (De La Lastra et al., 1972; Yun et al., 1987) suggests that the LH response to PMSG has two different components: a slight and prolonged elevation with the first peak independent of pituitary secretion and a precipitous second elevation of the pituitary-dependent surge. PMSG is known to possess an exceptionally high sialic acid content and a consequent slow clearance rate as well as a mixture of FSH- and LH-like activity when measured by bioassay
(Schams et al., 1978). The circulating half life of PMSG (30-36 hr) observed in the present experiment is consistent with the prolonged disappearance rate reported by others (Parlow and Ward, 1961; Sasamoto et al., 1972; Aggarwal and Papkoff, 1981). This estimation is considerably longer than the half life of endogenous gonadotropins which were reported to be 2.5 hr for FSH and 0.5 hr for LH in rats (Bogdanove and Gay, 1969). A study of neutralization with anti-PMSG serum demonstrated the active biological life of PMSG of 54 to 60 hr in mice (Sasamoto et al., 1972) and its circulating inactivation time of 36 hr in rats (Sasamoto and Kennan, 1973). On the basis of these chemical properties of PMSG and a significant cross-reaction between PMSG and rat LH antibody in the current assay system, one can conclude that the elevated serum LH around the time of the first peak is actually an intrinsic component of PMSG. Additionally, timing of the surge between 54 and 60 hr in the present experiment is well in agreement with the critical time of endogenous LH secretion established in numerous time-inhibition studies using various neuropharmacologic central nerve depressants (Sorrentino et al., 1972; Hillensjo et al., 1974) and hypophysectomy (Strauss and Meyer, 1962; Zarrow and Quinn, 1963).

It has previously been reported that in immature rats a low dose (3 IU) of PMSG initially stimulates a rapid follicular development during the first 36 hr prior to its inactivation in blood, and then by the next 25 hr endogenous gonadotropin is responsible for the maintenance of follicles to ensure final ovulation (Sasamoto and Kennan, 1973). In the present experiment, a superovulatory dose of PMSG resulted in the initial prolonged elevations of serum LH around the first peak and subsequent
suppression of the second, endogenous, LH surge as compared to control levels of serum LH. Therefore, in superovulated rats, the current observation of increased ovulatory response and ovarian weights as well as the previous findings of precocious ovulation as early as 24 hr (De La Lastra et al., 1972; Yun et al., 1987) reflect the effectiveness of PMSG-derived intrinsic gonadotropin for ovarian hyperstimulation. However, this interpretation does not rule out the involvement of endogenous gonadotropin secretion in the process of follicular maturation and ovulations even in superovulated rats, since the second elevation of serum LH remains higher than the preceding peak.

Several lines of evidence indicate that superovulatory regimens using exogenous gonadotropins inhibit the onset of endogenous LH surge and attenuate its magnitude in humans (Ferraretti et al., 1983; Messinis and Templeton, 1986) and monkeys (Littman and Hodgen, 1984). The results of present experiment are consistent with these findings, and provide further evidence on a nonprimate model. In rats, a superovulatory dose of PMSG significantly suppressed the endogenous LH surge without affecting the timing of its onset. This suppression has previously been attributed to the action(s) of inhibin-like protein and/or nonsteroidal ovarian factor(s) produced from hyperstimulated follicles which mediates directly a negative feedback of LH secretion (Ferraretti et al., 1983) or interferes with estrogen-mediated positive feedback (Littman and Hodgen, 1984). The substance responsible for this effect was further shown to be a factor of ovarian origin with a short circulating half-life, since bilateral ovariectomy restored normal pituitary responsiveness within 30 min (Schenken
and Hodgen, 1983). Therefore, it seems likely that a significant suppression of endogenous LH surge in superovulated rats is a reflection of hyperstimulation of multiple follicles by a superovulatory dose of PMSG. The factor(s) and its regulatory mechanism for the endogenous LH secretion in superovulated rats remain to be further clarified.

**Protocol 4.** Oocyte degeneration following superovulatory treatments with PMSG in immature rats has been associated with increased levels of ovarian androgens (Yun et al., 1987). In the present experiment, administration of an androgen antagonist (flutamide) after superovulatory dose (40 IU) of PMSG in immature rats significantly reduced oocyte degeneration, without influencing the ovulatory response, and embryo degeneration in preimplantation stage. Additionally, it improved the developmental potential of superovulated oocytes after fertilization, since there was a significant increase in the proportion of cleaved embryos.

The abnormalities in embryonic morphology and development resulting from superovulation have previously been ascribed to the abnormal secretion of ovarian steroid hormones (Miller and Armstrong, 1982) and the elevated levels of circulating steroids (Booth et al., 1975). The exposure to excessive amounts of estrogen and progesterone in vitro caused structural changes in fertilized embryos to block protein synthesis (Willmer, 1961). In addition, excess amounts of estrogen and progesterone resulted in cleavage arrest in mouse (Whitten, 1957; Kirkpatrick, 1971) and rabbit embryos (Daniel, 1964). In the present experiment, superovulation significantly elevated the circulating steroid hormones during the preimplantation period: 17β-estradiol on Day 2 and progesterone and
androgens on Days 2 to 5 of pregnancy. Flutamide treatment in superovulated rats significantly decreased androgen level, but this effect was limited on Day 2 without affecting the levels of 17β-estradiol and progesterone. Also, flutamide did not effectively prevent the early loss of preimplantation embryos resulting from superovulation and failed to improve embryo development beyond the 6-cell stage. Furthermore, its effects on preimplantation embryo development gradually decreased from Day 3. The partial effects of flutamide on preimplantation development may be accounted for by the steroid hormone response; it was insufficient to restore the prolonged elevation of androgens and progesterone resulting from superovulation (40 IU PMSG) to control levels (4 IU PMSG). Therefore, a significant decrease in cellular degeneration of preimplantation embryos and a significant increase in the proportion of cleaved embryos after flutamide treatment may be ascribed to a substantial reduction of abnormal oocytes before fertilization.

Timing of ovulation appears to be important to assess the occurrence of oocyte degeneration either within the follicle or in the oviduct and the impact of flutamide on this event. The present data for time-dependent ovulatory response by PMSG have shown a consistently steady state with an unchanged number of superovulated oocytes between 48 and 72 hr. These results are difficult to reconcile with the previous observations (Walton et al., 1983; Yun et al., 1987) indicating that superovulatory doses of PMSG induce multiple ovulations with an increased number of oocytes after 48 hr. It may be explained by batch to batch variation in the relative activity of FSH and LH in commercial PMSG.
preparations (Murphy et al., 1984). However, in view of the data of this experiment, actual timing of ovulation in superovulated rats appears to be complete by 48 hr, whereby the previously ovulated oocytes constitute a major population of degenerate oocytes observed between 48 and 72 hr. Aged oocytes after ovulation undergo a progressive deterioration within the oviduct which results in abnormalities of fertilization and early embryonic development (Austin, 1970; Longo, 1980). It is, therefore, likely that flutamide in superovulated rats may enhance the viability and survival of the previously ovulated oocytes within the oviduct presumably by altering steroid hormone response which may influence the oviductal environment. However, this interpretation does not discount the possibility that preovulatory steroid levels changed by flutamide may control the intrafollicular environment which in turn influences the viability and fertilizability of the oocytes. In the present experiment, flutamide substantially reduced androgens as well as 17β-estradiol in both ovarian tissue and serum, and subsequently resulted in a significant decrease in the proportion of degenerate oocytes and a marked decrease in early embryo degeneration. Also, in the previous experiment (Yun et al., 1989), alteration of follicular steroid contents (particularly androgens) following superovulatory treatment has been associated with atypical ovulations of meiotically premature or asynchronous oocytes which may contribute to the pool of abnormal oocytes prior to fertilization.

Mori et al. (1977) have suggested that androgens actively participate in the ovulatory process in rats treated with a low dose of PMSG. By neutralizing endogenous testosterone with its antiserum or by
inhibiting the preovulatory LH surge with a nonsteroidal androgen antagonist (hydroxyflutamide), the ovulatory response induced by low doses of PMSG was markedly reduced (Mori et al., 1977; Opavsky et al., 1987). In contrast, in the present experiment, the ovulatory response induced by a superovulatory dose of PMSG was not affected by flutamide administration. Possibly, sufficient LH-like activity in a superovulatory dose of PMSG may override the inhibitory effect of flutamide on the secretion of preovulatory LH. Interestingly, flutamide has recently been found to exert no effect on the modulation of LH surge in cycling adult rats (Jones et al., 1987). Differences in the effects of androgens on follicular development and function could most likely be ascribed to the differences in dosage, duration of action, or the particular androgen involved (Farookhi, 1985).

It has been reported that flutamide exerts its antiandrogenic effects on male sex organs by inhibition of androgen uptake and/or nuclear binding of androgens in vivo without other hormonal activities (Neri et al., 1972; Peets et al., 1974). However, in the present experiment, both ovarian and serum androgen levels in the flutamide-treated rats decreased significantly at 60 hr after PMSG and on Day 2 of pregnancy. Additionally, at 60 hr after PMSG, flutamide significantly decreased 17β-estradiol level. In a separate experiment, flutamide significantly decreased aromatizable androgenic steroids, androstenedione and testosterone but not a nonaromatizable androgen, 5α-dihydrotestosterone in serum samples obtained from 20 IU PMSG-treated rats. These results suggest that the decreased 17β-estradiol level by flutamide is due to the reduction of aromatizable androgens. In contrast, earlier study reported that flutamide failed to
alter the ovarian steroid content in immature rats treated with 5 IU PMSG (Peluso et al., 1980). The difference in response to flutamide on steroid hormone levels may be due to the different doses of PMSG employed. Superovulatory regimen may provide a different response to flutamide at the ovarian level by augmenting androgen production. The results of previous experiment have shown that superovulatory doses of PMSG significantly increased ovarian androgen levels, while a control dose significantly decreased the levels after 36 hr (Yun et al., 1987). It seems, therefore, that flutamide may exert its effects in superovulated rats by an alteration in steroid hormones, and conceivably by an inhibition of androgen binding in the ovary. This concept may explain the discrepancy in steroid hormone alterations observed on flutamide treatment: no significant difference at 48 hr and significant difference at 60 hr. The precise mechanism of flutamide action at the ovarian level in superovulated rats remains to be further elucidated.

From these observations, it can be concluded that 1) flutamide greatly reduces oocyte degeneration and cellular degeneration of preimplantation embryos in superovulated rats; 2) flutamide improves the developmental potential of superovulated oocytes as judged by a marked increase in the proportion of cleaved embryos; 3) flutamide appears to act by decreasing follicular androgen production following superovulation, but; 4) the effects of flutamide on embryo development gradually decreased in the progress of early pregnancy. The present results strongly suggest that the augmentation of ovarian androgen secretion preceding fertilization exerts a significant role in the perturbation of oocyte quality and subsequent embryo
development after superovulation. Further research is required to define the particular mechanisms in which androgens deleteriously affect oocyte quality and subsequent embryo development in superovulated rats.

Protocol 5. Steroid hormones secreted from PMSG-stimulated ovarian tissues in rats are thought to control the ovulatory response and oocyte morphology. Evidence for a role of androgens in the ovulatory process has been provided by the studies in which an androgen antagonist (flutamide) was found to interfere with spontaneous LH surge and ovulation in 4 IU PMSG-injected rats (Opavsky et al., 1987) and reduce oocyte degeneration resulting from superovulation with 40 IU PMSG in rats (Yun et al., 1988). These studies suggest the important roles of androgens in the control of ovarian function and oocyte quality in PMSG-treated rats; however, they do not exclude the possibility of involvement of other steroid hormones in the process of PMSG-induced ovulation and maintenance of oocyte quality. In the present experiment using the rats injected with either a control dose (4 IU) or a superovulatory dose (40 IU) of PMSG, administration of a progesterone antagonist (RU486) significantly reduced ovulatory response without affecting oocyte morphology, while an estrogen antagonist (tamoxifen) treatment was associated with a significant decrease in ovulatory response as well as an increase in the proportion of degenerate oocytes. The comparison between the treatments of RU486 and tamoxifen for the ovulation-inhibiting effects showed that the effectiveness in its performance is greater for tamoxifen in 4 IU PMSG-injected rats and for RU486 in 40 IU PMSG-injected rats.
The present observation of marked inhibition of ovulatory response after RU486 and tamoxifen in PMSG-injected rats suggests potential physiological roles of progesterone and estrogen in the process of PMSG-induced ovulation. Earlier studies (Costoff et al., 1974; Parker et al., 1976) demonstrated the patterns of preovulatory increases in circulating levels of these steroid hormones accompanied by preovulatory surges of FSH and LH in PMSG-injected rats. It appears that the rising level of preovulatory estrogens produced by PMSG exerts its stimulatory action at the hypothalamic level promoting the release of LHRH, and at the anterior pituitary to increase responsiveness to LHRH leading to gonadotropin secretion (Labrie et al., 1978). Also, increased levels of preovulatory progesterone may synergize with estrogens to facilitate gonadotropin secretion by lowering the threshold of the hypothalamus to estrogenic stimulus (Kawakami and Sawyer, 1959) and by increasing pituitary sensitivity to LHRH (McPherson and Mahesh, 1979). The positive feedback relationship between the steroid hormones and gonadotropin secretion could be further supported by the studies using antibodies or antagonists of 17β-estradiol and progesterone (Ferin et al., 1969; Labhsetwar, 1970; Rao and Mahesh, 1986) and employing these exogenous steroid hormones in ovariectomized rats (Legan et al., 1975; McPherson et al., 1975). Therefore, the integral actions of estrogens and progesterone at hypothalamic-pituitary axis seem to be necessary for the expression of spontaneous gonadotropin surge ensuring consequent ovulation. However, in the interpretation of this mechanism, it is important to point out the differences in relative contribution of each steroid hormone for the
stimulation of ovulatory response between the two groups of rats injected with different doses of PMSG. The results of present experiment in which tamoxifen was administered to 4 IU PMSG-injected rats at equipotent dose and to 40 IU PMSG-injected rats at even 2.5-fold higher dose compared to that of RU486 showed that the effectiveness of tamoxifen to inhibit ovulatory response was greater in 4 IU PMSG-injected rats but less in 40 IU PMSG-injected rats than that of RU486. This finding indicates that the major steroid hormone involved in PMSG-induced ovulation may be different depending upon the dose of this gonadotropin to stimulate ovaries: 17β-estradiol for a control dose-stimulation and progesterone for a superovulatory dose-stimulation.

It is probable that in PMSG-injected rats RU486 exerts its antagonism of the action of progesterone on hypothalamo-pituitary axis rather than on ovary, since it did not affect all other parameters of oocyte quality, tissue weight and steroid hormone secretion at ovarian level consistently in both groups of rats injected with 4 or 40 IU PMSG. This concept concerning the mode of action of RU486 is well in agreement with the study (Rao and Mahesh, 1986) in which RU486 treatment in 8 IU PMSG-injected immature rats and cycling adult rats significantly reduced the preovulatory surge levels of FSH and LH, but it had no effect on gonadotropin levels in immature rats 7 days after ovariectomy.

The mode of inhibitory action of tamoxifen on ovulatory response appears to be similar to that of RU486. A number of studies demonstrated that antiestrogenic effect of tamoxifen to inhibit the secretion of preovulatory gonadotropins is mediated principally via the
hypothalamo-pituitary axis rather than via ovary (Walpole, 1968; Labhsetwar, 1970; Billard and McDonald, 1973). However, in the interpretation for the whole mechanism of tamoxifen to modulate ovarian function, some caution is required. Current observation of a great increase in the proportion of degenerate oocytes and reduction in ovarian weight as well as marked changes in serum steroid hormones after treatment of tamoxifen may indicate the direct action of tamoxifen at ovarian level. The effects of tamoxifen on these parameters were consistent in both groups of rats injected with 4 or 40 IU PMSG. In the present experiment, tamoxifen treatment was associated with a significant increase in serum 17β-estradiol and/or serum androgens, and a significant decrease in serum progesterone in PMSG-injected rats. Evidence suggests the disparate action of estrogen antagonists including tamoxifen, clomiphene citrate and nafoxidine on cultured rat granulosa cells, whereby these antagonists substantially augmented FSH-stimulated estrogen production and inhibited FSH-stimulated progesterone production in a dose-related manner (Welsh et al., 1984). This observation has been ascribed to enhancement of aromatase activity and suppression of pregnenolone biosynthesis via direct mediation through granulosa cell binding sites specific for estrogen antagonists (Sgarlata et al., 1984; Welsh et al., 1984).

Early or sustained elevation of circulating preovulatory 17β-estradiol has been found to be embryotoxic presumably through a direct action on the preovulatory rat oocytes; the effect could be partially restored by antiserum to 17β-estradiol (Butcher and Pope, 1979). Furthermore, the oviducts under the influence of high levels of ovarian and
circulating 17β-estradiol have shown to secrete a low molecular weight substance to inhibit embryo development in mice (Cline et al., 1977) and rabbit (Stone and Hamner, 1977; Stone et al., 1977). On the other hand, it has been suggested that clomiphene citrate, related to tamoxifen in its chemical structure, may exert a direct estrogen-antagonistic effect on the intrafollicular oocytes to promote its degeneration and further to increase preimplantation embryo degeneration rates associated with reduction in developmental potential in mice (Laufer et al., 1983) and rabbits (Yoshimura et al., 1985). Similarly, an exposure of clomiphene citrate to cultured preovulatory follicles exhibited dose-dependent atretic-like changes in rats (Laufer et al., 1982). It seems, therefore, likely that the promotion of oocyte degeneration by tamoxifen treatment may be mediated by a direct antiestrogenic action of tamoxifen in the preovulatory follicles and/or by its elevation of circulating estrogens to produce hostile environment in the oviducts. However, in the present experiment, the primary factor(s) leading to tamoxifen-associated oocyte degeneration between the two groups of rats injected with different doses of PMSG could be different depending upon timing of ovulation. Time-course of ovulatory response to increasing doses of PMSG has previously been well described (Walton et al., 1983; Yun et al., 1987). Thus, in 4 IU PMSG-injected rats, the present observation of an increased proportion of degenerate oocytes after tamoxifen reflects postovulatory event at 72 hr after PMSG and suggests the impact of tamoxifen in the oviduct through its elevation of circulating 17β-estradiol as a primary factor. On the other hand, in 40 IU PMSG-injected rats, since ovulations were persistent between 60 and 72 hr after PMSG, the
tamoxifen-associated oocyte degeneration may be ascribed to both factors including the direct antiestrogenic action of tamoxifen in preovulatory follicles and its secondary impact in the oviduct. Additionally, the elevated level of circulating androgens by tamoxifen may be another contributing factor to the production of hostile oviductal environment hence promoting oocyte degeneration. It has previously been shown that a great reduction of oocyte degeneration by an androgen antagonist (flutamide) in superovulated rats with PMSG has been associated with significant decreases in circulating androgens as well as 17β-estradiol (Yun et al., 1988).

In summary, both a progesterone antagonist (RU486) and an estrogen antagonist (tamoxifen) exhibited inhibitory action of ovulation in PMSG-injected rats indicating the potential physiological roles of progesterone and estrogen for active participation in PMSG-induced ovulatory process. The relative ovulation-inhibiting effectiveness of the two steroid antagonists was different between the rats injected with two different doses of PMSG indicative of major steroid hormone involved in PMSG-induced ovulation: 17β-estradiol for a control dose-stimulation and progesterone for a superovulatory dose-stimulation. Additionally, the detrimental effect of tamoxifen on oocyte morphology has been demonstrated in PMSG-injected rats. It appears that tamoxifen-associated oocyte degeneration may be mediated by its known antiestrogenic action in preovulatory follicles and/or its elevation of circulating 17β-estradiol and/or androgens to produce hostile oviductal environment.
SUMMARY AND CONCLUSIONS

Ovulatory response, oocyte quality (gross morphology and nuclear maturation), and developmental potential of preimplantation embryos after administration of different doses (4, 20, or 40 IU) of PMSG to immature rats were studied with reference to the steroid hormone levels in ovarian tissues, peripheral blood, or follicular fluid and circulating LH response. In addition, the effects of an androgen antagonist (flutamide), a progesterone antagonist (RU486), and an estrogen antagonist (tamoxifen) on these parameters were also examined.

A low dose (4 IU) of PMSG induced a consistent ovulation of up to 15 oocytes per rat which was synchronized at 60-72 hr. However, superovulatory doses (20 and 40 IU) of PMSG induced prolonged multiple ovulations with maximum average counts of 51 to 58 oocytes per rat between 60 and 72 hr. Superovulatory treatments resulted in the first ovulation as early as 24 hr with two increases in the number of oocytes recovered from oviducts: one before 36 hr and the other after 48 hr. The early ovulation in superovulated rats could be, in part, ascribed to the exogenous LH activity of high doses of PMSG. The result of serum LH response to PMSG showed two distinct LH peaks in which the initial prolonged elevation of serum LH before 54 hr resulted from actual cross-reaction of the injected PMSG with LH antibody in the assay and was dose-dependent, while a precipitous second elevation between 54 and 60 hr resulted primarily from a pituitary-dependent LH surge. Additionally, the role of steroid hormones in the PMSG-induced ovulation was implicated in the experiments using the steroid antagonists. Administration of the RU486 or tamoxifen in the rats
injected with different doses (4 or 40 IU) of PMSG significantly interfered with ovulation between 60 and 72 hr after PMSG indicating active participation of both progesterone and estrogen in the gonadotropin-induced ovulation. The mode of inhibitory actions of these antagonists on ovulatory response was discussed. On the other hand, the treatment with flutamide in superovulated rats did not affect the ovulatory response.

Superovulatory treatments with PMSG caused a dose-dependent increase in the proportion of degenerate oocytes and a marked alteration of ovarian and serum steroid levels from 36 hr onwards. There was a significant positive correlation between the percentage of degenerate oocytes and androgen levels in both ovarian tissues and serum from 24 to 60 hr following superovulation. In the experiment to examine the steroid contents in follicular fluid and oocyte maturation, the results showed a close association between the incidence of meiotically aberrant oocytes and abnormal follicular steroidogenesis in superovulated rats. Superovulated oocytes displayed substantially different stages varying from prophase I to metaphase II between 24 and 72 hr, while the nuclear maturation of a majority of control oocytes recovered from the oviducts of 4 IU PMSG-injected rats at 72 hr was synchronized at metaphase II. The profiles of follicular steroid contents in superovulated rats were characterized by both a marked alteration of progesterone and particularly androgens and a consistent disruption of sequential changes in overall ratios of A/E, P/E, and P/A, which remained constant with no substantial changes as compared to control regimen. The pattern of each follicular steroid hormone in both control and superovulated rats closely resembled the change in ovarian
tissues and sera. It was, therefore, considered that (1) the maturation of follicle and oocyte in superovulated rats might occur independently of the normal physiological events by an altered intrafollicular steroid environment, and (2) a portion of superovulated oocytes with premature or asynchronous nuclear maturation might be susceptible to hostile oviductal environment presumably influenced secondarily by an altered follicular steroid response and contribute to the pool of a progressive increase in degenerate oocytes prior to fertilization. The possibility of atypical ovulations with abnormal oocytes derived from physiologically unfit follicles following superovulation was discussed.

To explore further the action of elevated androgens on the perturbation of oocyte quality in superovulated rats, the effects of flutamide in superovulated rats were examined on oocyte gross morphology and embryo development in preimplantation stage. Flutamide treatment after injection of a superovulatory dose of PMSG significantly reduced oocyte degeneration throughout the whole period examined between 48 and 72 hr, and cellular degeneration of preimplantation embryos. Additionally, it improved the developmental potential of superovulated oocytes after fertilization, since a great increase in the proportion of cleaved embryos was noticed. These effects exerted by flutamide were, in part, attributed to the decreased production of ovarian androgens (particularly aromatizable androgenic steroids, androstenedione and testosterone) which might influence the oviductal environment and in turn enhance the viability and survival of the previously ovulated oocytes within the oviduct. Flutamide treatment in superovulated rats significantly decreased ovarian and/or serum levels of
androgens at 60 hr and on Day 2 of pregnancy. The interpretation of the impact of flutamide on oocyte quality in the oviduct was based on the observation of actual timing of ovulation showing a steady state with an unchanged number of superovulated oocytes between 48 and 72 hr in this particular experiment. On the other hand, a gradual decrease in the effects of flutamide on embryo development was observed in the progress of early pregnancy, i.e. from Day 3 of pregnancy. Also, flutamide did not effectively prevent the early loss of preimplantation embryos resulting from superovulation and failed to improve embryo development beyond the 6-cell stage. The partial effects of flutamide on preimplantation embryo development could be accounted for by the steroid response to flutamide, since it was insufficient to restore the prolonged elevation of circulating androgens and progesterone resulting from superovulation to control levels. Pharmacological effect of flutamide to decrease androgen level was limited on Day 2 of pregnancy without affecting the levels of other steroid hormones. Finally, a significant decrease in cellular degeneration of preimplantation embryos and a significant increase in the proportion of cleaved embryos after flutamide treatment were ascribed principally to a substantial reduction of abnormal oocytes before fertilization.

The effects of RU486 and tamoxifen on oocyte morphology were also examined to understand the potential roles of progesterone and estrogen in the perturbation of superovulated oocyte quality. RU486 treatment, after administration of different doses of PMSG did not affect gross morphology of oocytes recovered from oviducts. However, it was found that tamoxifen treatment in both control and superovulated rats was associated with a
consistent increase in the proportion of degenerate oocytes. The promotion of oocyte degeneration after tamoxifen treatment was considered to be due to (1) its direct antiestrogenic action in the preovulatory follicles, since there was a significant reduction of ovarian weight 12 and 24 hr after treatment, (2) its secondary action to elevate circulating 17β-estradiol and/or androgens which might produce hostile oviductal environment. Tamoxifen treatment significantly increased serum levels of 17β-estradiol and/or androgens in both control and superovulated rats. The results of this experiment have indicated, in part, the involvement of estrogen, but not progesterone, to influence the oocyte quality in superovulated rats.

Collectively, the results of this study suggest that the impaired fertility seen after superovulatory treatments with PMSG results primarily from atypical ovulations of superovulated oocytes with premature or asynchronous nuclear maturation associated with abnormal follicular steroidogenesis by excessive gonadotropic stimulation. Elevated levels of ovarian and circulating androgens prior to fertilization were particularly implicated as a major factor causing the perturbation of oocyte quality and embryo development after superovulation. Finally, the process of multiple ovulations induced by superovulatory doses of PMSG appeared to involve the exogenous LH activity of PMSG as well as active participation of progesterone and estrogen.
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